Human Immunodeficiency Virus Infection of CD8 lymphocytes

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Declaration

I declare that the studies presented here are the result of my own independent investigation. This work has not been submitted for any other degree.

Alexandra Cochrane.

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Abstract

Human immunodeficiency virus-1 (HIV-1) infection causes a progressive failure of immune function which is fatal if untreated, and while current antiviral therapy slows the progression of disease no cure has been found. CD8 lymphocytes form the cytotoxic arm of the adaptive immune response and are vital in the control of HIV and other intracellular pathogens. HIV enters its target cell through binding to the CD4 molecule and therefore preferentially infects cells that express CD4 on their surface, such as CD4 lymphocytes and monocytes. CD8 lymphocytes are susceptible to HIV infection *in vitro*, and there is increasing evidence to suggest that they are also infected *in vivo*. Various mechanisms for the infection of CD8 lymphocytes have been proposed. Experiments using the thy/hu mouse model support export of intrathymically infected CD8 lymphocyte precursors, while recent *in vitro* data suggest that mature CD8 lymphocytes upregulate CD4 on activation (generating a $CD8^{bright}CD4^{dim}$ phenotype) and become susceptible to HIV infection.

To confirm that CD8 lymphocytes are infected with HIV *in vivo*, and to investigate the mechanism of infection, HIV long terminal repeat (LTR) DNA was quantified in CD8 lymphocyte subsets of known purity, isolated from the blood of 20 subjects with HIV infection. HIV LTR was demonstrated in CD8 lymphocytes of 18/20 subjects, and in the subjects with chronic infection the frequency of infection increased with disease progression. HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes was significantly more frequent (median 1197 HIV LTR copies / million cells) than that of CD8+CD4- lymphocytes (undetectable in 7/9 subjects, $p<0.01$), suggesting infection on activation rather than during intrathymic development. The level of infection in the $CD8^{bright}CD4^{dim}$ lymphocytes approached that in CD4 lymphocytes from the same subjects (median 3660 HIV LTR copies / million cells) and therefore could not be explained by CD4 lymphocyte contamination.

Given the high level of infection of $CD8^{bright}CD4^{dim}$ lymphocytes their prevalence and phenotype were assessed in 8 healthy and 16 HIV infected subjects. The proportion of CD8 lymphocytes with a $CD8^{bright}CD4^{dim}$ phenotype ranged from 0.3 to 3.4%, with no significant difference between HIV infected and healthy subjects. The majority displayed a CD45RA-ve CD27+ve (memory) phenotype, but in contrast to

the populations generated *in vitro*, the circulating population was not uniformly activated but rather comprised both activated and quiescent cells.

Thus HIV infected CD8 lymphocytes commonly circulate in HIV infected subjects, and are likely to be infected on activation rather than during intrathymic development. Targeted infection during activation could have a significant effect on the immune control of HIV and other infectious agents. Given that a proportion of the circulating $CD8^{bright}CD4^{dim}$ lymphocytes have a quiescent phenotype, they may act as a long lived proviral reservoir with implications for viral eradication.

Chapter 1: General introduction

In 1981 clinicians working in California were struck by the appearance of unusual infections and malignancies in young homosexual men (Masur *et al.*, 1981). By 1983 similar presentations were noted in intravenous drug users, haemophiliacs and Hiatian refugees, and it became apparent that an immunodeficiency syndrome of potentially epidemic proportions was spreading across the world. The new disease was termed Acquired Immunodeficiency Syndrome (AIDS), and a scientific quest to find the aetiological agent, treat the afflicted and control the epidemic began.

Over the last 23 years this quest has spawned an explosion of knowledge starting with discovery of the HIV virus as the causative agent of AIDS and progressing through the unravelling of molecular mechanisms to the design of novel antiretroviral agents. Through use of these drugs the immunodeficiency caused by HIV can now be held in abeyance for years, even decades, but HIV has not yet been eradicated from any infected individual, and the epidemic continues its uncontrolled spread with an estimated 40 million people now infected worldwide. The holy grail for the scientific community is the discovery of a vaccine to prevent transmission, but while huge inroads have been made into understanding the natural immune response to the virus and in developing novel strategies to maximise the immunogenicity of vaccine candidates, the prospect of a fully preventative vaccine remains elusive. In order to push forward from treatment to cure and from therapeutic vaccine to preventative vaccine we need to better understand the details of how HIV evades immune clearance, and how, over years of infection it slowly weakens the immune system causing the characteristic progressive immunodeficiency of AIDS.

This thesis explores one aspect of the complex interrelationship between HIV and the immune system: the infection of CD8 lymphocytes. CD8 lymphocytes, as the main effector arm of the adaptive immune system are pivotal in the antiviral immune

response and their infection and destruction could contribute to the failure of immune control of HIV as well as opportunistic infections. In addition long lived HIV infected CD8 lymphocytes could form a reservoir of transcriptionally inactive HIV provirus contributing to HIV persistence over years of antiretroviral therapy.

The first five sections of this introduction provide overviews of the discovery, classification, structure and life cycle of HIV, together with brief descriptions of the natural history of HIV disease and current therapeutics, and a summary of the maturation and function of T lymphocytes. This is followed by more detailed sections exploring topics directly relevant to the experiments performed. Section 1.6 reviews current thinking regarding the nature and cause of HIV induced immunodeficiency focusing on the numerical and functional decline in T lymphocytes. Sections 1.7 and 1.8 explore the importance of CD8 lymphocytes in the initial control of HIV replication, and their ultimate failure in the fight against disease progression. The role of proviral reservoirs for the persistence of HIV infection in the face of antiviral therapy is considered in section 1.9. Finally, in section 1.10 the evidence for and against HIV infection of CD8 lymphocytes is debated, and proposed mechanisms of infection presented. At the end of the introduction the scope of the thesis is presented, including a resume of the chapters to follow.

1.1 Discovery of HIV.

A single issue of science in 1983 contained a paper from Barre-Sinoussi, Chermann, Montagnier and associates at the Pasteur Institute reporting isolation of a retrovirus, subsequently termed lymphadenopathy associated virus (LAV), from a man with persistent lymphadenopathy syndrome (Barre Sinoussi *et al.*, 1983) and a report from Gallo and coworkers describing isolation of human T cell leukaemia virus (HTLV) from an individual with AIDS (Gallo *et al.*, 1983). Further work by these two groups led to the propagation of LAV and the isolation of additional HTLV like viruses (termed HTLV III) from adult and paediatric AIDS patients. At the same time Levy *et al.*, (1984), identified retroviruses termed AIDS associated retroviruses (ARV) which grew to high titre in peripheral blood mononuclear cells (PBMCs) and were

cytotoxic to CD4 lymphocytes. By 1986 these three prototype viruses were recognised as a single new retrovirus of the *Lentiviridae* family, and the new virus was termed human immunodeficiency virus (HIV).

Discovery of the causative agent lead to development of diagnostic tests and the true extent of the AIDS / HIV pandemic became apparent. The latest figures from the World Health Organisation put the current numbers of people infected at 40 million with the vast majority living in sub Saharan Africa.

1.2 The Origin and classification of HIV.

Genomic characteristics place HIV within the *Lentivirus* genus of the family *Retroviridae*. *Retroviridae* are characterised by their life cycle, in which the viral RNA genome is reversed transcribed into double stranded DNA that is then integrated into the host genome. All have the genes gag, pol and env, which encode essential structural proteins, enzymes and envelope proteins respectively. The current classification splits the family into seven genera, grouped into 'simple' viruses (alpha-, beta-, and gammaretroviruses whose genome contains only gag, pol and env, and 'complex' viruses (delta- and epsilonretroviruses, lenti- and spumaviruses) which contain additional genes encoding proteins with regulatory functions (Table 1-1, Figure 1-1). Lentiviruses were so named due to the long incubation period between infection and disease. They classically affect the haematopoetic and central nervous system.

Genus	Examples	Host species
Alpharetrovirus	Rous sarcoma virus (RSV)	Chicken
	Avian leukosis virus (ALV)	Birds
Betaretrovirus	Mason-Pfizer monkey virus (MPMV)	Primate
	Jaagsiekte sheep retrovirus (JSRV)	Sheep
	Mouse mammary tumour virus (MMTV)	Mouse
Gammaretrovirus	Murine leukaemia virus (MuLV)	Mouse
	Feline leukaemia virus (FeLV)	Cat
	Gibbon ape leukaemia virus (GaLV)	Gibbon
Deltaretrovirus	Bovine leukaemia virus (BLV)	Cow
	Human T-lymphotropic virus (HTLV) -1, -2	Human
Epsilonretrovirus	Walleye dermal sarcoma virus	Fish
Lentivirus	Human immunodeficiency virus (HIV)-1, -2	Human
	Simian immunodeficiency virus (SIV)	Primate
	Feline immunodeficiency virus (FIV)	Cat
	Equine infectious anaemia virus (EIAV)	Horse
	Visna/maedi virus (VMV)	Goat/Sheep
	caprine arthritis-encephalitis virus	Goat
	Bovine immmunodeficiency virus	Cattle
Spumavirus	Primate foamy virus (PFV)	Primates
	Feline foamy virus (FFV)	Cat
	Bovine foamy virus (BFV)	Cow

Table 1-1. Classification of *Retroviridae*.

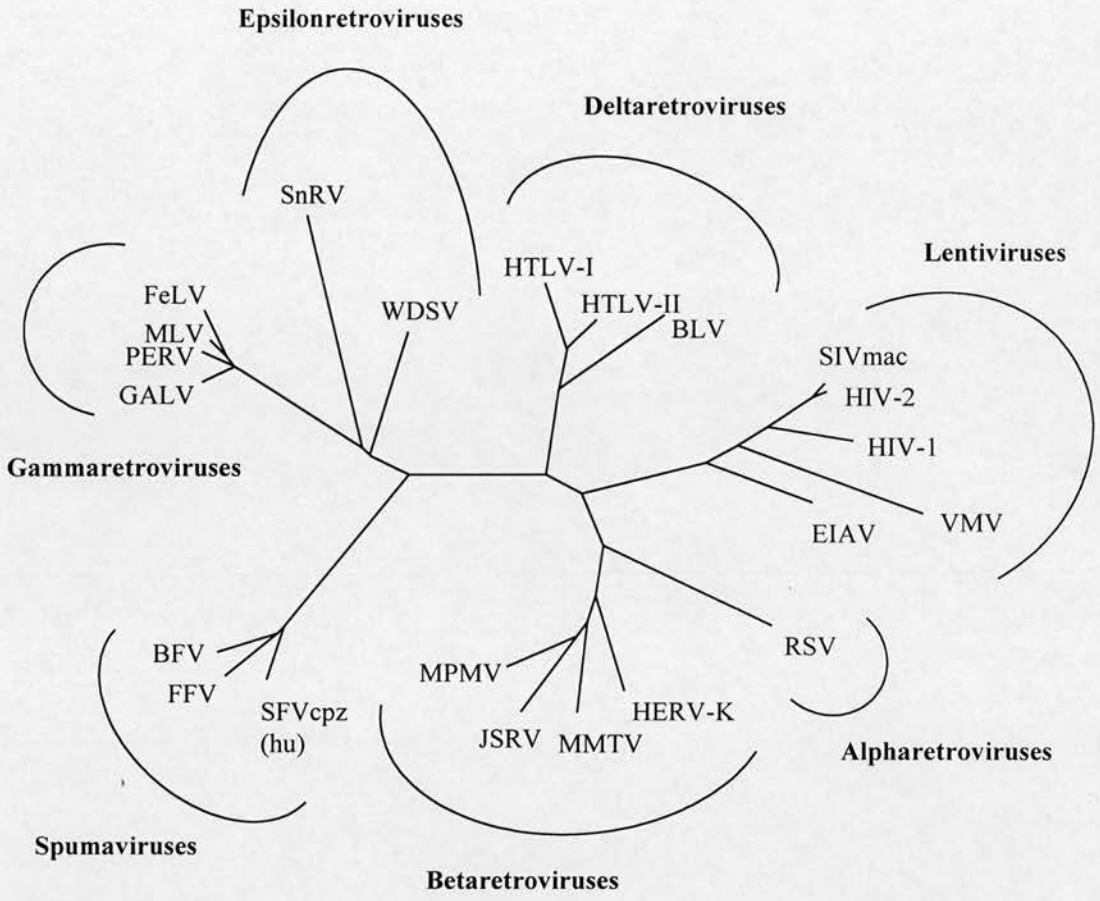


Figure 1-1. Phylogenetic relationship between retroviruses. A neighbour-joining tree based on the RT protein sequences of retroviruses. All seven retrovirus genera are labelled. See Table 1-1 for full virus names and descriptions. Phylogenetic tree courtesy of Dr. David Griffiths, Wohl Virion Centre, Windeyer Institute of Medical Sciences, University College London.

Extensive phylogenetic analysis of HIV isolates from across the globe have demonstrated two HIV viruses (HIV-1 and HIV-2), with HIV-1 being divided into three groups: M, O and N (standing for Main, Outlier and Non-M non-O) (Simon *et al.*, 1998)(Figure 1-2). HIV-1 group M is responsible for the global pandemic, and is further divided into 11 subtypes or 'clades' on the basis of sequence analysis. The huge diversity of subtypes in Africa, and the phylogenetic relationship between HIV and simian immunodeficiency virus (SIV) indicates that HIV originated following transmission of SIV from monkeys into humans in Sub-saharan Africa. At least two separate transmission events from chimpanzees are thought to be responsible for the

M and O clades of HIV-1 (Gao *et al.*, 1999), while HIV-2 is thought to have originated from transmission from the sooty mangabey (Hirsch *et al.*, 1999b).

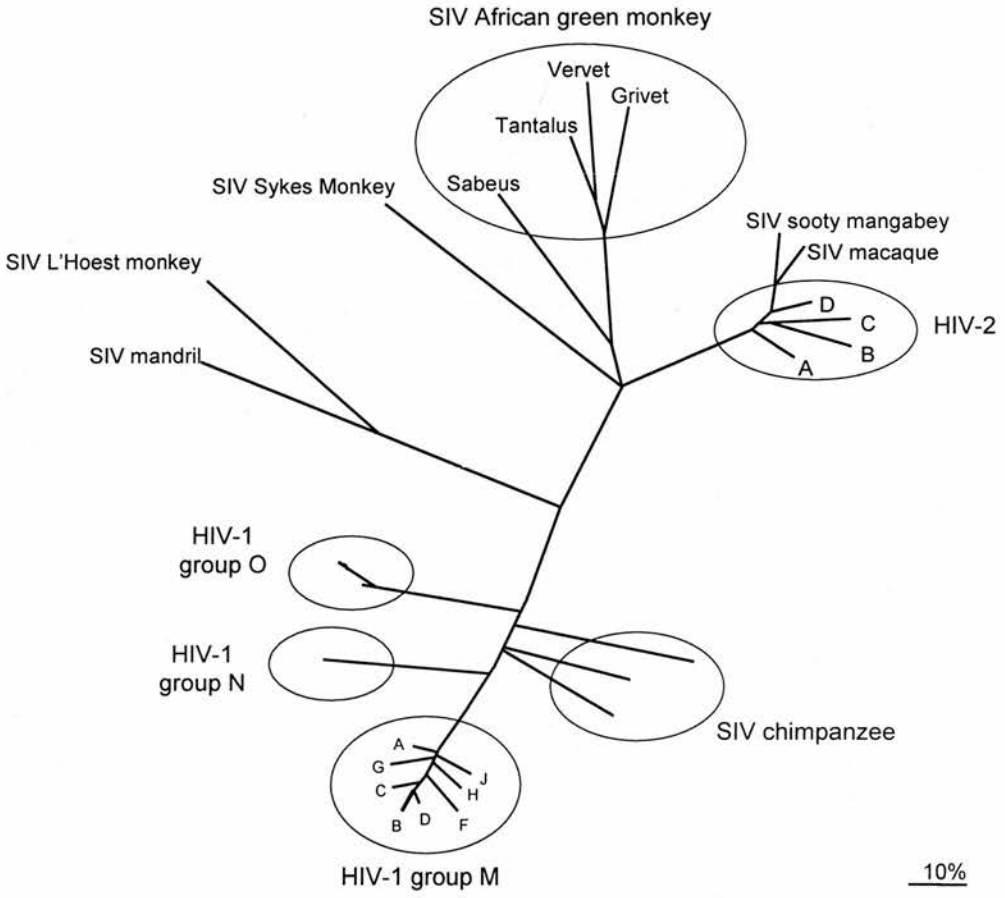


Figure 1-2. Phylogenetic relationship between HIV-1, HIV-2 and SIV. The positions of HIV-1 subgroups M, N and O are shown in relation to HIV-2 and various SIV isolates. Vervet, Grivet, Sabeus and Tantalus are subspecies of African green monkeys. Adapted from (Reeves & Doms, 2002)

1.3 The Structure, Genomic Organisation and Life Cycle of HIV

1.3.1 Structure

Mature HIV virions are between 80 and 100nm in diameter, and are composed of a lipid bilayer envelope surrounding a cone shaped protein core containing the viral RNA. The lipid bilayer envelope is acquired from the plasma membrane of the infected cell during the budding process and contains the virally encoded envelope glycoproteins: transmembrane glycoprotein gp41 and surface glycoprotein gp120. Heterodimers of gp120 are non-covalently linked to gp41 to form a trimeric 'spike' which is vital for attachment and entry to the host cell. Directly inside the lipid bilayer lies the p17 matrix protein which ensures structural integrity and plays a role in the insertion of envelope proteins into the mature virion. The core is composed of the viral capsid protein p24 containing two identical copies of the positive sense RNA genome closely associated with reverse transcriptase (a viral DNA dependent RNA polymerase), and the nucleocapsid protein p7. Also within the core lie the virally encoded proteins Protease, Integrase, virion infectivity factor (Vif), Nef, Viral protein R (Vpr), p6 and possibly Tat.

1.3.2 Genomic organisation.

The 9.8 kilobase HIV RNA genome encodes nine open reading frames flanked by long terminal repeats. The three largest open reading frames (gag, pol and env) are found in all *Retroviridae*, and encode nucleocapsid core proteins, enzymes (reverse transcriptase, protease and integrase) and envelope glycoproteins respectively. The remaining six open reading frames encode two regulatory proteins (Tat and Rev) involved in the regulation of transcription and splicing, and four accessory proteins Vif, Vpr, Vpu, and Nef, (Figure 1-3).

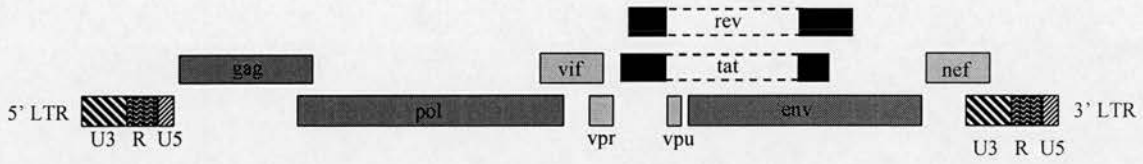


Figure 1-3. Genomic organisation of HIV and processing of viral proteins. Dark grey shading denotes the three genes common to all retroviruses (gag, pol and env), encoding structural proteins and essential enzymes; black shading indicates regulatory genes; very pale grey represents accessory genes. The LTR domains are represented as hatched boxes. Figure adapted with permission from Dr. Samantha Willey, Laboratory of Clinical and Molecular Virology, University of Edinburgh.

1.3.3 Life Cycle.

At its simplest, the HIV life cycle can be summarised as entry into the host cell, reverse transcription of the viral RNA with integration of newly formed viral DNA into the host genome, use of the host cell machinery to transcribe and translate the viral DNA, and finally post translational processing of viral proteins, followed by formation and budding of the new virions (Figure 1-4). The process of cell entry is of particular relevance to the question of HIV infection of CD8 lymphocytes, and the detail of reverse transcription and integration is of relevance to the quantification of HIV. These aspects are considered in more detail.

HIV enters target cells through a process of attachment followed by fusion. Attachment occurs through binding of the trimeric gp120 on the virion surface to a CD4 molecule on the target cell. This binding induces a confirmational change in gp120 exposing a chemokine receptor binding site (Kwong *et al.*, 1998). Binding at this site causes a conformational change in gp41 leading to the projection of three peptide fusion domains through the cell membrane (Chan *et al.*, 1997). Fusion of the target cell and virion membranes then occurs with release of the viral core into the target cell (Doms & Trono, 2000). Up to twelve chemokine receptors have been shown to act as coreceptors *in vitro*, but only CCR5 and CXCR4 are frequently used *in vivo* (Doms & Trono, 2000). Of note, transmitted virus generally uses CCR5 (and is known as R5 virus), and subsequently diversifies to use both CCR5 and CXCR4 (and is termed X4 virus). This switch often occurs at the time of progression to

AIDS but whether it is causative remains unknown (Scarlatti *et al.*, 1997). HIV can also enter cells through endocytosis, but this does not result in productive infection (Greene & Peterlin, 2002).

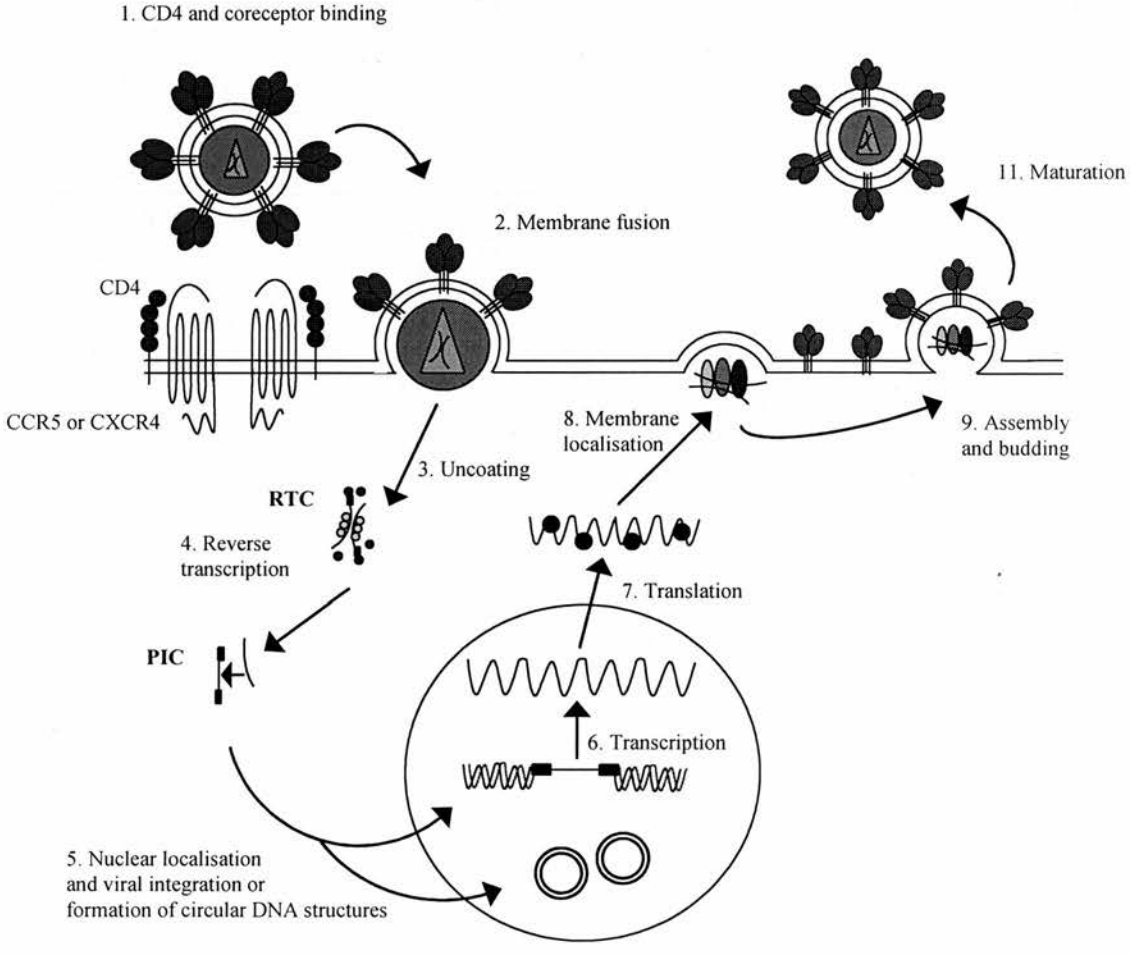


Figure 1-4. Life cycle of HIV. The major stages in the life cycle of HIV from receptor binding to virus particle budding. RTC, reverse transcription complex; PIC, pre-integration complex. Figure adapted with permission from Dr. Samantha Willey, Laboratory of Clinical and Molecular Virology, University of Edinburgh.

Following entry into the host cell viral uncoating occurs, the reverse transcription complex (RTC) is formed and reverse transcription generates the HIV preintegration

complex (PIC) containing double stranded viral cDNA. The error prone nature of reverse transcriptase and RNA polymerase II, which lack proof reading ability, leads to a high mutation rate estimated at $3x10^{-5}$ per *in vivo* replication cycle (Mansky & Temin, 1995). The PIC then travels through the nuclear membrane (an active process which allows HIV to infect non-dividing cells) and either integrates successfully into the host genome forming an HIV provirus (resulting in a productive infection), or forms one or many circular DNA structures (Bukrinsky *et al.*, 1993). In common with all retroviruses, the HIV virion contains two RNA copies producing a single provirus. (Hu & Temin, 1990). HIV DNA can integrate anywhere in the host genome and in the presence of Tat efficient transcription of the viral genome is initiated.

1.3.4 HIV cytopathicity

HIV can lead to cell death through a number of means, including direct cytotoxic effects on infected cells, cytotoxic T lymphocyte (CTL) mediated killing of infected cells, and bystander cell death. Direct cytotoxic effects have been observed in cell culture and include induction of cell fusion (Lifson *et al.*, 1986a; Lifson *et al.*, 1986b) accumulation of viral DNA (Shaw *et al.*, 1984), loss of membrane integrity (Cloyd & Lynn, 1991), interference with cellular RNA processing (Agy *et al.*, 1990), and alteration in cytokine production (Macchia *et al.*, 1991; Meyaard *et al.*, 1994b). These effects tend to be more severe when the infecting virus originates from an individual with advanced disease, and virus isolates can be divided into 'syncytium inducing' (SI) and non-syncytium inducing (NSI) on the basis of their ablity to induce cell fusion in culture (Tateno & Levy, 1988).

CTL mediated killing of HIV infected cells is covered in sections 1.6.7.4 and 1.7. Bystander cell death is a process by which uninfected cells are killed, and has been consistently observed in SIV infected macaques and HIV infected subjects (Meyaard *et al.*, 1992; Li *et al.*, 2005). Bystander cell death is generally mediated by apoptosis, which is more frequent amongst CD8 than CD4 lymphocytes (Lewis *et al.*, 1994). Apoptosis can be caused by the HIV proteins nef and env. Nef has been shown to induce FAS-ligand expression on infected cells resulting in apoptosis of

neighbouring uninfected cells expressing FAS (Geleziunas *et al.*, 2001). Studies using nef deficient SIV demonstrated that both CD4 lymphocytes and CD8 lymphocytes were killed through this mechanism (Xu *et al.*, 1997). Env has been shown to upregulate TNFα expression by macrophages, and TNF receptor expression by CD8 lymphocytes. Subsequent contact between these two cell types resulted in CD8 lymphocyte apoptosis by a TNFα dependent mechanism (Herbein *et al.*, 1998). Death by apoptosis is also a natural consequence of lymphocyte activation, and much of the increased apoptosis in HIV may be due to activation induced cell death.

In addition to causing cell death, HIV can interfere with target cell function. This is further discussed in relation to lymphocytes in sections 1.6.7.3 and 1.6.7.5.

1.4 The Natural history and current treatment of HIV infection.

1.4.1 Primary infection

HIV is efficiently transmitted by sexual contact, inoculation via contaminated needles or blood products, and from mother to child both perinatally and via breast milk. During sexual contact the virus enters the mucosal layer where it is able to infect CD4 lymphocytes and tissue macrophages. It is then transported to the draining lymph nodes both in infected lymphocytes and by attachment to DC-SIGN expressed on the surface of dendritic cells (Geijtenbeek *et al.*, 2000). Rapid multiplication and dispersal follows and within days of infection virus can be detected at high levels in serum (Reimann *et al.*, 1994; Kahn & Walker, 1998), and found in diverse anatomical sites (Davis *et al.*, 1992). The immune response to infection is covered in more detail elsewhere (section 1.7). Briefly, the virus elicits a specific anti HIV cytotoxic T lymphocyte response that reverses the rising viraemia, bringing the viral load down to the so called 'set point'. Specific anti HIV antibodies follow and are usually detectable in plasma within 12 weeks of infection (Busch *et al.*, 1995). During this period of high viraemia and early immune responses the majority of infected individuals will experience a mild 'seroconversion illness' with symptoms such as fever, rash and lymphadenopathy.

1.4.2 Chronic infection

Following seroconversion the infected individual enters an asymptomatic period lasting on average 6 to 8 years during which the viral load climbs and the CD4 lymphocyte count drops progressively (Lifson *et al.*, 1994). During this period the total CD8 lymphocyte count remains stable but the naïve CD8 lymphocyte count drops in parallel with the CD4 lymphocytes (Figure 1-5). When the CD4 lymphocyte count reaches a level of 200 – 350 the infected individual becomes increasingly susceptible to pathogens such as *Mycobacterium tuberculosis*, varicella-zoster virus, Candida species and *Streptococcus pneumoniae*. The onset of AIDS is defined clinically through diagnosis of an AIDS defining illness (Castro *et al.*, 1992). The individual becomes vulnerable to opportunistic pathogens such as *Pneumocystis jirovecii*, as well as malignancies, HIV associated wasting and dementia. Without intervention AIDS is usually followed by death within 4 years (Michael & Burke, 1991). Typical changes in HIV viral load and CD4 and CD8 lymphocyte counts during the course of infection are shown schematically (Figure 1-5).

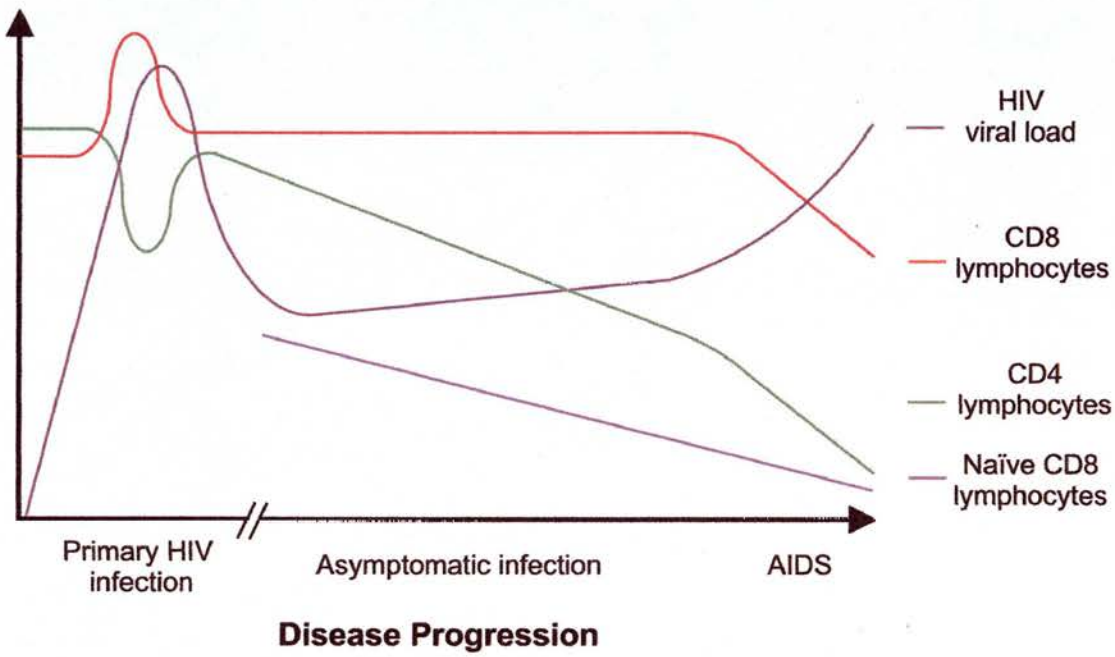


Figure 1-5. Natural history of HIV infection. Schematic diagram of the natural history of HIV infection during primary and chronic phases, indicating relative changes in CD4 and CD8 lymphocyte counts and plasma viral load. The relative slopes of the CD4 and naïve CD8 lymphocyte decline are approximate and based on published data (Roederer *et al.*, 1995).

1.4.3 Long-term non-progressors.

Approximately 8 – 10% of individuals infected with HIV do not follow the normal course of progression to AIDS, instead they maintain a normal CD4 lymphocyte count and low viral load without recourse to antiviral treatment. A huge research effort has been expended looking for the key to immune resilience in these individuals, and many diverse factors, (reviewed in (Mikhail *et al.*, 2003), have been found to associate with non-progression to a greater or lesser extent. While defective or slowly replicating viral strains, or heterozygosity for a CCR5 deletion are sometimes implicated (Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995), host factors modulating the T lymphocyte response appear to be the major determinants. Thus long term non-progressors characteristically have a broad CTL response, a T_H1 cytokine expression pattern (Clerici *et al.*, 1993), and a persistently strong CD8 non-cytotoxic antiviral response (Levy, 1993). In addition the HLA types B27 and B-57 are represented at greater frequency in long term non-progressors than in the general population (Kaslow *et al.*, 1996a).

1.4.4 Current therapy and vaccine prospects.

1.4.4.1 **Antiviral therapy**

Antiviral therapy for HIV infection began with the approval of zidovudine (AZT), a nucleoside analogue reverse transcriptase inhibitor (NRTI), in 1987. It soon became apparent that the effect of monotherapy with AZT was shortlived, and the addition of a second NRTI, didanosine (ddI), resulted in only limited improvement as the virus rapidly developed resistance to the two drugs. HIV management was revolutionized in 1995 with the advent of triple combination therapy, in which a protease inhibitor or non-nucleoside reverse transcriptase inhibitor was used in combination with at least two other antiretrovirals. These combinations, often refered to as highly active antiretroviral therapy (HAART), brought HIV viral load down to undetectable levels in a significant proportion of treatment naïve patients (Hirsch *et al.*, 1999a), allowed

immune reconstitution (Autran *et al.*, 1997; Li *et al.*, 1998) and provided a significant survival benefit (Hammer *et al.*, 1997). More recently fusion inhibitors have been added to the anti HIV pharmacopoeia, providing an additional option for heavily pretreated patients. Even with the use of triple drug combinations resistance remains a major problem, and transmission of drug resistant strains now occurs in 20% of new infections in the UK (Pillay, 2004).

1.4.4.2 **Immunomodulatory therapy.**

In the late 1990s it became clear that, due to reservoirs of HIV within brain, semen and quiescent T lymphocytes, and low levels of viral replication even in the presence of HAART, a lifetime of viral suppression with HAART will not eradicate the virus (see section 1.6.4). Thus patients currently face lifelong antiviral medication with the associated problems of viral resistance, side effects and pill burden, and the attention of the research community has refocused on therapies to 'flush out' the latent virus through activation of quiescent lymphocytes, and to stimulate the immune response to successfully control low levels of viraemia. Strategies to achieve these aims include structured treatment interruptions (reviewed in(Lori *et al.*, 2002), therapeutic vaccination (reviewed in (Lisziewicz *et al.*, 2003) and immune stimulants such as hydroxyurea and IL-2.

Structured treatment interruption involves repeated withdrawal of antiviral treatment, allowing rebound viraemia and consequent stimulation of the immune response. In macaques with acute SIV infection this approach led to development of strong HIV specific T cell responses associated with complete control of viraemia (Lori *et al.*, 2000). Similar results were achieved in HIV infected individuals when treated within three months of infection (Lisziewicz *et al.*, 1999; Rosenberg *et al.*, 2000), but these successes have not generalized to subjects with chronic disease (Dybul *et al.*, 2003).

Three major types of therapeutic vaccines have been trailed: subunit vaccines containing HIV-1 proteins (both recombinant proteins (Goebel *et al.*, 1999) and whole inactivated virus (Churdboonchart *et al.*, 1998)), recombinant viral vectors encoding HIV-1 proteins (Jin *et al.*, 2002), and DNA vaccines (Macgregor *et al.*, 1998). Many have demonstrated immunogenicity but none have yet achieved

clinical benefit. A novel strategy in which a DNA vaccine is applied directly to slightly exfoliated skin aims to stimulate expression of HIV proteins by Langerhans cells which would then mature to dendritic cells and present the antigens to naïve T cells in draining lymph nodes. This approach has shown promise in combination with HAART in macaques chronically infected with an HIV/SIV construct (SHIV) (Lisziewicz *et al.*, 2005).

Il-2 and hydroxyurea have also undergone trials, with the hope that they will both improve immune function and activate latently infected T lymphocytes thus rendering them susceptible to viral cytotoxicity or CTL attack (Kulkosky *et al.*, 2002). Initial results demonstrated that IL-2 causes a marked increase in CD4 lymphocyte count (Arno *et al.*, 1999) and a more rapid loss of HIV infected cells (Lafeuillade *et al.*, 2001). In addition, IL-2 treatment in acute HIV infection leads to augmentation of the CD8 lymphocyte non-cytotoxic anti-HIV response (Martinez-Marino *et al.*, 2004). The combination of IL-2 and therapeutic vaccination is showing promise, leading to increased breadth of HIV specific CD8 lymphocytes (Levy *et al.*, 2005).

1.4.4.3 **Vaccines**

Development of a protective vaccine is the holy grail of HIV research. In general the research community is pessimistic about the likelihood for developing a truly protective vaccine within a reasonable timeframe, with generation of a vaccine that decreases the efficiency of transmission being a more realistic goal. The magnitude of the task is highlighted by a case of superinfection of an HIV infected subject, even when mounting a good CTL response against epitopes present in the superinfecting virus (Altfeld *et al.*, 2002). In addition, passive immunization using immune sera did not protect macaques challenged with SIV (Kent *et al.*, 1994). Optimists argue that in cases of superinfection the subjects' immune system is compromised by HIV and that this would not be the case in vaccine induced immunity. Indeed, evidence from highly exposed seronegative subjects, such as sex workers or the partners of HIV infected individuals, suggest that a degree of protective immunity against systemic infection can be developed.

Traditionally the stimulation of a good neutralizing antibody has been sufficient for a vaccine, but HIV has evolved to resist antibody attack. The exposed envelope proteins are highly glycosylated and flexible (Reitter *et al.*, 1998). They vary by up to 10% of amino acids within an individual, and any conserved regions are hidden within clefts. In addition functional virions are outnumbered 1000:1 by non-functional particles displaying aberrant envelope structures. Finally the envelope proteins are surrounded by an array of host cell derived proteins and serum proteins, all inhibiting the attachment of antibody (Wyatt & Sodroski, 1998). Despite these viral adaptations, following an extensive search in sera from HIV infected subjects five monoclonal antibodies have been found that can neutralize a broad range of primary clade B HIV isolates (Moore *et al.*, 2001). These antibodies are directed against the conserved regions of HIV envelope protein, some of which are only exposed after CD4 binding. They can protect SCID mice reconstituted with human lymphoid cells against challenge with HIV (Gauduin *et al.*, 1997), and can protect monkeys against SIV/HIV (SHIV) hybrid virus (Mascola *et al.*, 2000; Hofmann-Lehmann *et al.*, 2001). However, the titres required are higher than are generally achieved by immunization, and the technicalities of presenting the relevant protein conformations to the immune system are considerable.

Difficulties generating neutralizing antibodies, together with evidence demonstrating the importance of CTLs in control of HIV (see section 1.7) have shifted the focus of vaccine research towards T cell mediated immunity. T cells cannot prevent infection as they are active against infected cells, but they can limit the extent of infection and prevent disease, as demonstrated in mice vaccinated against influenza and paramyxoviruses (Ulmer *et al.*, 1998). Generating a CTL response broad enough to be effective against all the common circulating HIV variants, in host populations of varying HLA makeup, is a considerable challenge. Extensive investment has been dedicated to finding the optimum number and type of epitopes to include, and the optimal strategy for delivery (Boyer *et al.* 2000).

There has also been considerable progress in development of the international cooperation required for large scale efficacy testing of candidate vaccines. The first HIV vaccine to undergo efficacy trials, AIDSVAX (VaxGen's recombinant gp120

vaccine candidate), demonstrated no protective effect, but there are now over 30 further candidates undergoing phase I and phase II trials, with clinical sites in Africa, Asia, Latin America, Europe and the USA (details available from The International Aids Vaccine Initiative; http://www.iavi.org).

1.5 T lymphocytes

1.5.1 T lymphocyte function

T lymphocytes form the backbone of the adaptive immune response. All bear the T cell receptor (TCR) which acts in conjunction with either CD4 or CD8 molecules to bind antigen presented in conjunction with MHC on the surface of cells. CD4 lymphocytes have been referred to as the 'conductors of the immune system orchestra', reflecting their ability to influence the behaviour of other components of the immune system. They recognise antigen presented in conjunction with MHC class II by professional antigen presenting cells, and provide co-stimulatory signals to B lymphocytes, macrophages and CD8 lymphocytes. These signals may be provided through direct cell contact or through the secretion of cytokines. Depending on the nature of the antigen presentation and the cytokine environment, CD4 lymphocytes may generate a T_H1 or a T_H2 response. Though there is considerable overlap, the T_H1 response is dominated by release of IFN-γ, IL-2 and IL-3, and promotes a cellular response to the pathogen, while the T_H2 response is typified by release of IL-4 and IL-5 and promotes a humoral response.

The classic function of CD8 lymphocytes is to combat intracellular pathogens through release of cytotoxic granules triggered by recognition of foreign peptide presented in conjunction with MHC class I on the surface of infected cells. Additional functions include secretion of antiviral cytokines such as IFNγ, and β chemokines, and recognition and destruction of neoplastic cells.

1.5.2 T lymphocyte maturation

T lymphocytes develop in the thymus from bone marrow derived precursors. Within thymocytes the TCR gene segments rearrange such that each cell expresses a particular variant of TCR which will bind a limited range of antigen. The thymocytes then undergo a process of positive then negative selection to retain only cells that recognise self MHC molecules but do not react against self antigens. They are then released into the circulation as antigen naïve T lymphocytes. At this stage their function is to patrol the lymphoreticular system awaiting contact with appropriate activation signals. The thymus is at its most active in the neonate and rapidly wanes through childhood. Some activity is retained in adult life but this continues to decrease with age (Mackall *et al.*, 1995). The naïve T cell population can be replenished from the thymus, allowing generation of cells with novel TCR specificities, or through regenerative proliferation of existent naïve T lymphocytes. Observations of T lymphocyte regeneration following myeloablative therapy show that while naïve CD8 lymphocytes can de repopulated by proliferation of circulating cells, naïve CD4 lymphocyte reconstitution is thymus dependent (Hakim *et al.*, 1997; Mackall *et al.*, 1997).

Naïve T lymphocytes are activated by interaction with professional antigen presenting cells, and require two signals: the recognition of a foreign antigen bound to a self MHC molecule, and a second co-stimulatory signal. This process is usefully referred to as priming to differentiate it from the activation of more mature T cells. Antigen binding to the T cell receptor in the absence of co-stimulation leads to anergy, a process that may be involved in HIV induced immunodeficiency. The co-stimulatory signal is often provided by binding of CD28 on the lymphocyte to B7 molecules on the APC. CD8 lymphocytes require a stronger co-stimulatory signal than CD4 lymphocytes, and the level of B7 expression on the APC may be insufficient. In this case the APC must present the same antigen simultaneously to a naïve CD8 lymphocyte and a primed CD4 lymphocyte. The CD4 lymphocyte then induces upregulation of B7 molecules on the APC, increasing the strength of the co-stimulatory signal. Thus, efficient priming of naïve CD8 lymphocytes requires CD4 lymphocyte help (Matloubian *et al.*, 1994).

Priming leads to clonal expansion. Primed T lymphocytes stimulate their own proliferation through synthesis of the growth factor IL-2 and its receptor, with a single cell being capable of dividing two or three times a day. After 4 or 5 days of proliferation the progeny cells differentiate into short lived effector lymphocytes. In general the CD8 lymphocytes will differentiate into cytotoxic cells while CD4 lymphocytes develop helper capability, but there is some overlap of function and CD4 lymphocytes with cytotoxic potential are expanded in HIV infection (Appay *et al.*, 2002b). On re-encounter with cognate antigen these effector cells are able to function rapidly without the need for a co-stimulatory signal. A minority of the primed T lymphocytes remain as memory cells after the effector response has subsided.

For CD8 lymphocytes a linear model of differentiation has been proposed (further discussed in section 3.1.1) in which primed cells first differentiate into cells with high proliferative potential (termed memory cells) and then progress to terminally differentiated effector cells loosing their ability to proliferate, but gaining cytotoxic capability (Hamann *et al.*, 1997; Kern *et al.*, 1999; Hamann *et al.*, 1999a; Champagne *et al.*, 2001). Memory CD8 lymphocytes have been subclassified into central memory and effector memory depending on their expression of homing molecules. Thus central memory cells express CCR7 and CD62L and home to lymph nodes, while effector memory express neither of these two molecules and home to sites of inflammation in the tissues (Sallusto *et al.*, 1999). In well controlled chronic viral infections the virus specific CD8 lymphocytes tend to have a memory phenotype, but differentiate to the effector phenotype if viraemia rebounds (Zhang *et al.*, 2003).

1.5.3 $CD8^{bright}CD4^{dim}$ lymphocytes

Until the 1980s it was generally accepted that CD4 and CD8 expression on mature T lymphocytes were mutually exclusive. With the advent of two colour flow cytometry in 1985 a small population of $CD4^{+}CD8^{+}$ lymphocytes were observed making up 2-3% of PBMCs in the blood of healthy subjects (Blue *et al.*, 1985). These cells were negative for the thymocyte marker T6 and had the size, granularity

and expression profiles of activated cells. Further analysis demonstrated two distinct subpopulations: $CD8^{bright}CD4^{dim}$ which were associated with acute viral infection and thought to originate from CD8 lymphocytes, and $CD4^{bright}CD8^{dim}$ which were proposed variants of CD4 lymphocytes (Ortolani *et al.*, 1993).

Conclusive evidence that mature CD8 lymphocytes can upregulate CD4 to generate a $CD8^{bright}CD4^{dim}$ phenotype came in 1998 with two papers in which highly purified CD8 lymphocytes responded to *in vitro* stimulation by production of CD4 mRNA and cell surface CD4 expression (Flamand *et al.*, 1998) (Kitchen *et al.*, 1998). The type of CD8 lymphocyte most likely to upregulate CD4, the nature of the signal required and the phenotype of the resulting $CD4^{bright}CD8^{dim}$ lymphocytes are discussed in more detail in section 5.1.

These findings led to the hypothesis that naïve CD8 lymphocytes *in vivo* may temporarily upregulate CD4 expression following antigen specific activation, generating the $CD8^{bright}CD4^{dim}$ lymphocytes observed in the circulation. This proposal is consistent with the observation that $CD8^{bright}CD4^{dim}$ lymphocytes *in vivo* are more likely to display an antigen experienced phenotype than their CD4-ve counterparts (Imlach *et al.*, 2001; Nascimbeni *et al.*, 2004).

1.6 HIV induced immunodeficiency.

The hallmark of HIV infection is immunodeficiency and yet our understanding of its nature is patchy with even the most fundamental aspects, such as the cause of the CD4 lymphocyte decline, remaining controversial. Studies of other immunodeficiency viruses in their natural hosts, including SIV in Sooty mangabeys and African green monkeys, indicate persistent infection but little disease, and even a species switch, as occurred with HIV-2 has not induced the devastating immune collapse typical of HIV-1. The immunodeficiency of HIV-1 is pervasive, affecting both the innate and adaptive responses, and compromising the function of macrophages (Kedzierska *et al.*, 2003), dendritic cells (Macatonia *et al.*, 1990) and natural killer cells (Cai *et al.*, 1990) as well as B and T lymphocytes (Schnittman *et al.*, 1986). While acknowledging the importance of all these aspects, this review is restricted to T lymphocyte immunodeficiency, a topic directly relevant to the

potential role that HIV infection of CD8 lymphocytes may play in HIV pathogenesis. The effect of HIV infection on T lymphocyte numbers, turnover, distribution, activation and function, are described first, and then the mechanisms behind the defects observed are explored. The dynamics and function of HIV specific T lymphocytes are discussed separately (1.7 and 1.8). Before moving on to specifics it is salutary to recall the degree to which advances in our understanding of HIV have been paralleled by advances in immunology. When HIV was first isolated the T cell receptor had not been discovered, CD8 lymphocytes were not known to recognise viral peptides, and the distinction between T_H1 and T_H2 subsets of CD4 lymphocytes had not been made. Thus early observations often have to be re-evaluated in the light of more recent advances.

1.6.1 T lymphocyte numbers.

1.6.1.1 **Primary infection.**

The recent demonstration that acute HIV infection causes a rapid and profound depletion of antigen experienced CD4 lymphocytes has revolutionised our understanding of HIV immunopathology. Studies in SIV infected macaques demonstrated that by day 10 post infection 60 – 80% of antigen experienced CD4 lymphocytes were lost from blood, lymph node and gastrointestinal mucosa (Mansky, 2000; Mattapallil *et al.*, 2005). The depletion was most profound for cells expressing CCR5 with a $CD95^{hi}CCR7-$ (effector) phenotype, which are preferentially located in mucosal tissue (Veazey *et al.*, 1998; Veazey *et al.*, 2000). Gut mucosae together with gut associated lymphoid tissue (GALT) contain 60% of the total body lymphocytes and have been identified as the major site of CD4 lymphocyte loss in both acute and chronic SIV infection (Veazey *et al.*, 1998; Veazey *et al.*, 2000; Mansky, 2000; Mattapallil *et al.*, 2005; Li *et al.*, 2005). That HIV has a similar effect in humans was suggested by demonstration of marked CD4 lymphocyte loss in jejunal biopsies from two subjects with PHI (Guadalupe *et al.*, 2003), and confirmed in two papers published together in September last year (Mehandru *et al.*, 2004; Brenchley *et al.*, 2004b). Mehandru *et al.*, (2004), studied colonic biopsies from 27 HIV infected subjects (13 with PHI) and 10 uninfected controls. The proportion of

mucosal mononuclear cells expressing CD4 and CCR5 was significantly reduced in primary HIV infection, and immunohistochemistry of biopsy sections demonstrated that while the inductive lymphoid sites (comprising the B cell follicle and surrounding T cell zone) contained normal numbers of CD4 lymphocytes, approximately 2/3 of these cells were lost in effector sites (lamina propria). In a recent review of T cell dynamics Douek argues that these early events leave a profound and long-lasting impact on the immune system that may set the stage for the subsequent progressive immunodeficiency (Douek *et al.*, 2003).

1.6.1.2 **Chronic infection**

In contrast to the rapid depletion of activated antigen experienced CD4 lymphocytes in the acute phase, during the chronic phase peripheral blood CD4 lymphocyte counts drop very slowly. Both memory and naïve subsets decline, but the naïve subset declines more rapidly, such that the proportion of CD4 lymphocytes that are naïve drops from 50% in healthy subjects to approximately 25% in subjects with AIDS. The profound deficiency of CD4 lymphocytes in GI tissue in primary infection is maintained into chronic infection, with a trend towards ongoing decline (Mehandru *et al.*, 2004; Brenchley *et al.*, 2004b).

Total CD8 lymphocyte numbers remain stable until late stages of disease, but this stability masks changes in CD8 lymphocyte subsets. If antigen naïve and experienced cells are considered separately then a steady decline in the naïve subset is observed which parallels the decline in naïve CD4 lymphocytes (

Figure 1-5) (Roederer *et al.*, 1995). HIV infection of CD8 lymphocytes may contribute to this decline, but as discussed in section 1.6.6 the relationship between infection and depletion is complex.

1.6.2 T lymphocyte redistribution

The movement of T cells between blood, lymphoid and extralymphoid compartments is controlled by the interaction of an array of cellular adhesion molecules and homing receptors, and their counterparts on endothelial surfaces. Immune activation of T lymphocytes classically causes downregulation of lymph node homing molecules

such as CD62L and CD27, and an increase in tissue homing molecules, resulting in redistribution of cells away from the lymphoreticular system and into the tissues. Given that only 2% of total body CD4 lymphocytes reside in the circulation, small shifts in homing to lymph node or tissues can lead to large changes in the circulating population (Trepel, 1974; Zhang *et al.*, 1998). HIV infection results in prolonged immune activation (described in section 1.6.3) and it was soon recognised that much of the CD4 lymphocyte decline observed in acute and chronic HIV infection was due to cell redistribution rather than loss (Bucy *et al.*, 1999). The extent of the redistribution was revealed by studies examining immune reconstitution following HAART. The early rapid rise in the antigen experienced CD4 lymphocyte population was attributed to resolution of the HIV induced redistribution, with the slower rise in the naïve population attributed to regeneration (Parker *et al.*, 1998). A recent study of T cell dynamics in multiple tissues biopsied from SIV infected macaques demonstrated that population shifts play little part in the massive loss of CD4 lymphocytes seen in acute infection (Mattapallil *et al.*, 2005).

1.6.3 T lymphocyte activation.

HIV infection results not only in activation of responding lymphocytes as would be expected for any acute viral infection, but also profound generalised immune activation which extends into the chronic phase. In acute infection this generalised activation is evidenced by increased expression of activation and proliferation markers including CD38 and Ki67 not only on HIV specific, but also EBV, CMV and influenza specific CD8 lymphocytes (Trepel, 1974; Doisne *et al.*, 2004). Evidence of continuing generalised activation into the chronic phase of disease is provided by increased expression of activation markers including CD25, HLA-DR, CD38 and Ki-67 on αβ-and γδ-T lymphocytes from blood and lymph nodes (Norazmi *et al.*, 1995; Peakman *et al.*, 1995; Benito *et al.*, 1997; Brenchley *et al.*, 2004b). There is also increased turnover of both CD4 and CD8 lymphocytes (Sachsenberg *et al.*, 1998; Hazenberg *et al.*, 2000b), polyclonal activation of B lymphocytes, and in SIV infection, increased proliferation of B lymphocytes and NK cells (Mohri *et al.*, 1998).

1.6.4 T lymphocyte turnover

An early estimate of T lymphocyte turnover in HIV was published in Nature in 1995 (Wei *et al.*, 1995; Ho *et al.*, 1995). The authors used rates of CD4 lymphocyte recovery post HAART to postulate that, in subjects with asymptomatic chronic infection, CD4 lymphocyte turnover was grossly elevated, with an estimated 5% of the total CD4 lymphocyte population ($1 - 2 \times 10^9$ cells) dying and being replaced on a daily basis. The authors estimated that this represented a 78 fold increase on normal CD4 lymphocyte turnover. This hypothesis spawned a plethora of studies, (reviewed in (Johnson, 2000), assessing the effect of HIV on CD4 lymphocyte turnover. Conflicting results were reported, largely because 'turnover' is not an exact concept but an amalgam of production, destruction and life span, and its rate was inferred using a number of inexact correlates. Thus the observation that telomere lengths of CD4 lymphocytes in HIV infected persons remained stable over time suggested no increase in turnover (Wolthers *et al.*, 1996), but this conclusion was contradicted by studies of Ki67 expression (Sachsenberg *et al.*, 1998), and by bromodeoxyuridine (BrdU) labelling in SIV infected macaques (Mohri *et al.*, 1998). Development of ^{2}H-glucose labelling allowed direct measurement of cell proliferation and destruction rates in healthy human volunteers and was a huge step forward for the field. Following the publication of two studies using this technique it is generally agreed that HIV infection leads to a moderate increase in turnover, with a 3 – 6 fold increase in fractional CD4 lymphocyte proliferation and destruction rates (Hellerstein *et al.*, 1999; Mohri *et al.*, 2001).

1.6.5 Lymph node architecture

HIV disease leads to progressive destruction of lymph node architecture. (Schacker *et al.*, 2002). The degree of destruction has been shown to correlate with the extent of effector T lymphocyte infiltration of the lymph node, and is thought to be immune mediated (Brenchley *et al.*, 2004b).

1.6.6 Cause of T cell depletion

It is tempting to speculate that HIV infection of CD8 lymphocytes may cause the depletion in naïve CD8 lymphocyte numbers. However, as elaborated in this section, the relationship between HIV infection of CD4 lymphocytes and CD4 lymphopaenia is still hotly debated, so any ideas regarding the causality of CD8 lymphocyte depletion must be viewed in the light of this ongoing debate.

It has been proposed that the massive loss of antigen experienced $CD4^+$ lymphocytes in primary HIV or SIV infection is caused by infection of large numbers of activated cells (activated through recent interaction with gut associated microbes) followed by viral cytopathicity or CD8 mediated killing (Veazey *et al.*, 1998; Veazey *et al.*, 2000; Veazey & Lackner, 2003; Mattapallil *et al.*, 2005). This hypothesis is supported by the very high numbers of productively infected CD4 lymphocytes within the gut (Mattapallil *et al.*, 2005), together with *in vitro* demonstrations of HIV cytopathicity, and CD8 directed lysis of HIV infected cells. However, detailed immunohistochemical studies of SIV and HIV infected GI tissue have shown that the areas of maximum infection are the inductive lymphoid tissue while the maximal CD4 cell loss occurs in the effector tissue of the lamina propria (Mehandru *et al.*, 2004; Li *et al.*, 2005), leading authors to suggest that cell loss is largely bystander cell death. In keeping with this proposal Li *et al.*, (2005), found peak levels of infection could only account for 20% of the total CD4 lymphocyte depletion. In addition markers of apoptosis as well as expression of Fas and Fas-ligand were increased in the effector but not the inductive tissue, and this expression correlated in time with the peak destruction of CD4 lymphocytes.

The cause of the T lymphocyte loss during chronic infection is clearly more complicated as the subsets in sharpest decline (ie naïve CD4 and CD8 lymphocytes) are not major targets for productive infection with CCR5 tropic virus. The main theories regarding the cause of depletion are reviewed below.

1.6.6.1 **Accelerated destruction.**

The first theory to address the cause of chronic CD4 lymphocyte decline was the 'tap and drain' or 'accelerated destruction' model introduced by Ho and Wei (Wei *et al.*,

1995; Ho *et al.*, 1995). This theory contains two basic premises, first that the increased CD4 lymphocyte turnover is driven by excess destruction mediated by viral cytotoxicity or CTL killing of infected cells with the increased proliferation being a homeostatic response to lymphopaenia, and second that the progressive CD4 lymphocyte decline is caused by exhaustion of the regenerative capacity of the immune system in the face of prolonged high turnover.

Since the theory was proposed the degree of T lymphocyte turnover has been revised downwards, but the two basic premises of the theory retain support (Mohri *et al.*, 2001). HIV infected CD4 lymphocytes, while present at a very low frequency in blood (1/1000 – 1/10,000 PBMCs) (Simmonds *et al.*, 1990; Chun *et al.*, 1997) are detected at higher levels in lymph node (Pantaleo *et al.*, 1993) and GI tissue (Mohri *et al.*, 2001; Anton *et al.*, 2003; Mehandru *et al.*, 2004), and it remains conceivable that the observed level of increased turnover may be driven by HIV induced destruction.

However, viral cytotoxicity and CTL killing preferentially effect antigen experienced cells, while the cell population in rapid decline is the naïve subset. Ho *et al.*, (1995), did not clearly delineate the proposed mechanism linking these two events, but possibilities include homeostatic movement of cells from the naïve compartment (Picker *et al.*, 2004). Clearly in subjects carrying a CXCR4 tropic virus naïve lymphocytes may be depleted by direct infection (Blaak *et al.*, 2000). The question of CD8 lymphocyte depletion is not addressed by proponents of this theory, but viral cytotoxicity and CTL mediated killing could lead to increased turnover of HIV infected CD8 lymphocytes.

1.6.6.2 **Immune activation**

Others argue that the increased lymphocyte turnover is fuelled by generalised immune activation which causes lymphocyte proliferation, naturally followed by death through activation induced cell death (AICD) (Hazenberg *et al.*, 2000a; Hazenberg *et al.*, 2000b; Grossman *et al.*, 2002). To investigate this issue, Kovacs *et al.*, (2001), used BrdU labelling in HIV infected people. They found that HAART reduces the fraction of proliferating CD4 lymphocytes but not their half life

suggesting that proliferation rather than premature destruction is the driving force behind increased turnover.

Proponents of the immune activation theory postulate that activation not only causes increased cell turnover, but also leads to the decline in CD4 lymphocyte count and disease pregression. This link is supportive by correlative evidence, by animal model studies and by studies of IL-2 therapy. Thus, markers of immune activation correlate more strongly with CD4 lymphocyte decline than does viral load (Sousa *et al.*, 2002), and are predictive of disease progression. Also, in its natural hosts (Sooty Mangabeys and African Green Monkeys) SIV replicates to high copy number and is cytopathic to infected cells, but causes no increased cell turnover and no immunodeficiency (Chakrabarti *et al.*, 2000; Broussard *et al.*, 2001). In contrast, in Macaques the viraemia excites high levels immune activation, increased cell turnover and a progressive lymphopaenia (Kaur *et al.*, 1998). Finally, IL-2 induced CD4 lymphocyte expansion in HIV infected subjects is associated with decreased Ki67 expression and decreased turnover (Sereti *et al.*, 2004).

The chronic immune activation of HIV affects both CD4 and CD8 lymphocytes (see section 1.6.3) but to date the mechanism by which activation could cause naïve CD4 and CD8 lymphocyte decline has not been demonstrated. Possibilities include: sustained activation of naïve lymphocytes which then enter the memory pool; induction of an anergic state in which lymphocytes become less sensitive to signals promoting regenerative proliferation resulting in reduced steady state numbers of resting cells (Grossman *et al.*, 2002); or disruption of T cell regeneration by collagen deposition in lymph tissue (Brenchley *et al.*, 2004b).

1.6.6.3 **Regenerative failure.**

The third major theory of CD4 lymphocyte decline, known as the regenerative failure model, proposes that HIV induced failure of production rather than increased destruction is paramount. It was championed by Hellerstein and McCune following a cross sectional study in which the absolute production rate of CD4 lymphocytes per ml of blood was not significantly different in HIV infected compared to uninfected subjects (despite significant lymphopaenia), but was increased 3 fold in subjects

following 12 weeks of HAART treatment. (Hellerstein *et al.*, 1999; McCune *et al.*, 2000). Supportive findings include a link between speed of progression and measures of thymic potential (Touloumi & Hatzakis, 2000; Teixeira *et al.*, 2001), but attempts to show a direct link between CD4 lymphocyte decline and reduced thymic output have been compromised by methodological issues (Hazenberg *et al.*, 2000a). Against this theory, Hellerstein and McCune's findings regarding the effect of HAART were contradicted by two longitudinal studies (Mohri *et al.*, 2001)(Kovacs *et al.*, 2001), both of which found that treatment causes a definite reduction in CD4 lymphocyte proliferation.

Of interest, in the SIV infected macaques, animals that progressed rapidly to disease had both marked acute phase loss of memory CD4 lymphocytes, and a progressive failure of proliferation of the remaining memory cells. In contrast, animals that progressed slowly were able to maintain the residual memory CD4 lymphocyte population by ongoing proliferation (Picker *et al.*, 2004). The cause of the failure of proliferation was not addressed, but this finding does suggest that while accelerated destruction in the acute phase is paramount, failure of production may be critical in disease progression.

1.6.7 T lymphocyte function

In addition to the decline in numbers of CD4 and CD8 lymphocytes in HIV infected persons, there is evidence that those that remain do not function optimally. Studies comparing LTNP versus rapid progressors suggest that T lymphocyte function may be crucial in curbing disease progression, (Migueles *et al.*, 2002). Again, it is easy to assume that abnormalities in CD8 lymphocyte function may be due to HIV infection of CD8 lymphocytes, but, as was seen for the decline in lymphocyte numbers, the lessons from CD4 lymphocytes indicate that direct infection is only one of many possible causes of dysfunction. In this section an overview of the methods used to measure lymphocyte function is given, highlighting aspects of relevance to HIV. This is followed by a brief review of HIV induced dysfunction of CD4 lymphocytes, and then CD8 lymphocytes are considered in more detail. The proposed mechanisms behind the defects are also presented.

1.6.7.1 **Measuring T lymphocyte function.**

The contribution of T lymphocyte dysfunction to HIV induced immunodeficiency has been tested *ex vivo* using a variety of approaches which involve measuring the response of the cell to a stimulus (Allen & Watkins, 2001). The methods are often technically demanding and poorly standardised and have produced a myriad of conflicting results in the field of HIV immunopathology. Importantly the vast majority of investigations have been performed on lymphocytes isolated from blood, while the effector functions are largely delivered in peripheral tissue or lymph nodes. Many of the older methods require culture for up to a week and thus could under-estimate the function of cells from HIV infected subjects which are prone to apoptosis in culture. Teasing out the true cause of the responses observed and relating these back to *in vivo* function is challenging. The main assays used and the number of days culture they require are summarised in Table 1-2.

T cell attribute of interest	Method	Approx. time in culture	Response measured	Factors contributing to the measured response
Proliferation[a]	T lymphocytes stimulated and cultured in presence of ^{3}H thymidine.	7days	^{3}H thymidine uptake	i) Number of precursors available capable of proliferating to the stimulus given; ii) Number and rate of divisions from each precursor cell. iii) Ability to survive and proliferate under culture conditions
Effector cytotoxicity[a]	Freshly isolated T lymphocytes cultured with ^{51}Cr labelled target cells.	4 hours	Chromium release.	Number of fully differentiated cytotoxic T lymphocytes able to lyse the target cells.
Precursor cytotoxicity[a]	T lymphocytes cultured with stimulus then added to ^{51}Cr labelled target cells.	7 days	Chromium release.	i) Number of cytotoxic T lymphocytes precursors present in sample that are capable of differentiating in response to the stimulus given, and capable of lysing the target cells. ii) Ability to survive and proliferate under culture conditions
Number of antigen specific CD8 lymphocytes	Tetramer	none	Binding to tetramer	Number of cells present able to bind to the tetramer used.
Cytokine secretion	Bulk cytokine assays	7 days	Amount of cytokine secreted into solution.	i) Number of cells able to secrete cytokine of interest in response to stimulus given. ii) Amount of cytokine secreted by each cell iii) Ability to survive in culture
Cytokine secretion	ELISPOT	1 day	Number of cells secreting a cytokine in response to a stimulus	i) Number of cells present able to secrete cytokine in response to the given stimulus. ii) Ability to survive in culture
Cytokine secretion	Intracellular cytokine staining	4 hours	Number and phenotype of cells secreting one or many cytokines in response to a stimulus.	Number of cells present able to secrete cytokine in response to the given stimulus.

Table 1-2. Evaluating T-cell responses. (a) These assays can be performed at limiting dilution allowing estimation of the proportion of cells that have the given attribute.

1.6.7.2 CD4 lymphocyte function in HIV infection

A number of studies in the 1980s demonstrated that CD4 lymphocyte function, as measured by proliferative response, IL-2 production or CD4 lymphocyte dependent B lymphocyte function, was reduced in HIV infected subjects (Lane *et al.*, 1983; Miedema *et al.*, 1988; Clerici *et al.*, 1989; Weimer *et al.*, 1989; Shearer & Clerici, 1991). Loss of function was observed in CD4 lymphocytes derived from blood and

lymph node (Jobe *et al.*, 1999). These deficiencies were initially reported in subjects with AIDS, and were assumed to be caused by a profound loss of CD4 lymphocyte numbers, but it soon became apparent that some functions were defective in earlier stages of disease when CD4 lymphocyte numbers were only slightly reduced. In a classic experiment Clerici et al.compared proliferation and IL-2 production of stimulated peripheral blood lymphocytes in 96 HIV infected subjects with 70 controls. There was a clear loss of both proliferation and IL-2 production in the HIV infected group which was present at the asymptomatic stage of disease and became more profound over time. Thus 84% of asymptomatic HIV infected subjects lacked response to recall antigens (tetanus toxoid and influenza), 30% lacked response to recall antigens and HLA alloantigens and 14% did not respond to recall antigen, alloantigen or mitogen (PHA). In subjects with more progressive disease none of the 22 subjects responded to recall antigens, 36% responded to alloantigen and 64% had no response to any of the stimuli (Clerici *et al.*, 1989). Other investigators have not found proliferative defects (Gurley *et al.*, 1989). The range of proliferative responses reported may be due to the smaller sample size of some later studies (Gurley *et al.*, 1989), or may reflect the different intensities of stimulus used by different investigators, as the loss of proliferation can be overcome by strong stimulation (Meyaard *et al.*, 1994a).

Significant loss of proliferative response can occur early in the course of HIV infection. In subjects tested within three months of seroconversion the majority had no detectable response to *Candida* or tetanus, and had decreased response to PHA. These responses recovered to some extent in the next 12 months but did not return to the levels seen in uninfected controls (Musey *et al.*, 1999).

Where proliferative defects are observed, they are generally reversed with effective antiretroviral therapy. Thus (Angel *et al.*, 1998), found the majority of treated subjects recovered their proliferative response to mitogen (PHA) and in a separate trial proliferation to recall antigen also improved (Kelleher *et al.*, 1996).

Measurement of antigen specific CD4 lymphocyte function using intracellular cytokine staining has produced a very different picture. Rather than the expected progressive loss of function, HIV infected subjects demonstrated a largely intact

response with the fraction of CD4 lymphocytes responding to CMV being enhanced, while that responding to mumps was decreased (Waldrop *et al.*, 1997; Pitcher *et al.*, 1999). These findings led the authors to suggest that the early loss of antigen experienced cells, and the increased lymphocyte turnover generates an environment in which lymphocytes responding to frequently encountered antigen out compete memory lymphocytes generated during past events. This would explain the preserved immune control of opportunistic infection until late stages of disease.

A number of investigators found that the antigen specific CD4 lymphocyte response in HIV infected subjects was dominated by secretion of IFN-γ and TNF-α, with little secretion of IL-2 or IL-4 (Waldrop *et al.*, 1997; Sieg *et al.*, 2001). As proliferation requires IL-2, this cytokine pattern may explain the conflict between these results and those measuring response in terms of proliferation. Alternatively the length of *in vitro* culture required for the different methodologies may be instrumental (see section 1.6.7.1. T lymphocytes from HIV infected subjects are known to be prone to apoptosis *in vitro*, and the 5 –6 day culture used in proliferation assays may have resulted in the death of many of the effector cells. In contrast, the intracellular cytokine staining protocol requires only a 6 hour stimulation.

1.6.7.3 **Mechanism of decreased CD4 lymphocyte function.**

A number of mechanisms could contribute to the decline in CD4 lymphocyte function, including subset imbalance, toxic effects of the virus and its proteins, changes in the cytokine environment and defective antigen presentation.

Direct cytotoxic effect. The cytotoxic effects of HIV on infected CD4 lymphocytes are described in section 1.3.4. While these effects often result in cell death, they can also impair function in CD4 lymphocytes that are infected but not killed. Given the low frequency of infection of CD4 lymphocytes, especially during the asymptomatic stages, these mechanisms alone could not account for all the loss of function observed. However they may be of particular relevance to the loss of HIV specific CD4 lymphocyte function, as these cells are preferentially infected (Douek *et al.*, 2002).

CD4 lymphocyte subset imbalance. Van Noelson *et al.,* (1990), demonstrated selective loss of activated memory ($CD4^+CD29^+$) as opposed to naïve CD4 lymphocytes in asymptomatic HIV infected subjects with normal CD4 lymphocyte counts. The authors noted that many of the functional abnormalities observed early in infection, including lack of response to recall antigen, to anti-CD3 or anti-CD2 monoclonal antibodies, and lack of helper activity could all be explained by this subset imbalance. Subsequent more detailed phenotypic analyses have confirmed the loss of memory cells, now thought to result largely from massive loss of CCR5 expressing (largely activated memory) CD4 lymphocytes during primary infection. (Schnittman *et al.*, 1990; Veazey *et al.*, 1998; Veazey *et al.*, 2000; Veazey & Lackner, 2003; Mattapallil *et al.*, 2005). Of relevance decreased frequencies of alloreactive precursor cells, and decreased proliferative responses per alloreactive cell, have been demonstrated in both CD45RA+ and CD45RO+ T lymphocyte populations. This was interpreted to indicate loss of function within the naïve and memory subsets, indicating that subset imbalance alone is unlikely to account for all the dysfunction observed (Meyaard *et al.*, 1994a).

Effect of circulating viral proteins. Viral envelope proteins are secreted from infected cells (Tas *et al.*, 1988) and have been noted to have immunosuppressive effects on T lymphocytes (Chanh *et al.*, 1988; Diamond *et al.*, 1988; Gurley *et al.*, 1989; Oyaizu *et al.*, 1990; Quintana *et al.*, 2005). This effect is thought to be mediated through interactions with cell surface receptors causing dysfunction of activation pathways (Rosenstein *et al.*, 1990), and alteration of cytokine secretion (Oyaizu *et al.*, 1990). Toxic effects such as induction of cell fusion and membrane defects could also be important (gp120) (Lifson *et al.*, 1986a; Miller *et al.*, 1993). In addition Gag can affect intracellular function through binding to cyclophillins (Luban *et al.*, 1993) while Tat has been shown to reduce the proliferative response of CD4 lymphocytes to recall antigens (Seth *et al.*, 2000).

Cytokines. The cytokine milieu is central to the fine balance of immune function, and there is increasing evidence that HIV causes disturbance of both cytokine production and the response to cytokines (reviewed in (Kedzierska & Crowe, 2001). For example, HIV infection of macrophages *in vitro* leads to increased secretion of

TNFα, a cytokine that can be toxic to T cells (Merrill *et al.*, 1989), and in SIV or SHIV infected macaques CD4 lymphocytes are deficient in cytokine expression (McKay *et al.*, 2003). In addition, PBMCs from HIV infected subjects are deficient in IL-12 production, a cytokine important for T cell proliferation and IFN-γ production (Marshall *et al.*, 1999). Inappropriate cytokine secretion may also induce the shift from a T_H1 to a T_H2 type immune response, an event which is thought to be crucial for progression to AIDS (Clerici & Shearer, 1997), and could contribute to the generation of T lymphocyte anergy.

Defective antigen presentation and anergy. Dendritic cells are responsible for transporting antigen to lymph nodes and activating naïve T lymphocytes. HIV is known to bind strongly to DC-SIGN (Geijtenbeek *et al.*, 2000), a cell surface molecule involved in the activation process. This could result in antigen being presented to the TCR in the absence of costimulation, causing naïve T cells to become anergized rather than activated. Similarly restimulation of antigen experienced T cells by macrophages may be compromised by HIV induced downregulation of MHC class II and the costimulatory molecules CD80 and CD86 (Clerici *et al.*, 1991; Dudhane *et al.*, 1996). Anergy could also be induced by low affinity TCR interactions caused by the presentation of subtly mutated epitopes or downregulation of TCR expression (Trimble & Lieberman, 1998). Anergic cells compete with naïve cells for antigen and are thus powerful inhibitors of a specific T lymphocyte response. HIV induction of anergy is an attractive hypothesis explaining many of the features of CD4 and CD8 lymphocyte function (Maier *et al.*, 1998), but direct evidence is lacking.

1.6.7.4 **CD8 lymphocyte function in HIV infection.**

Of interest to this thesis is whether HIV infected individuals have compromised CD8 lymphocyte function, which could be caused by direct infection of CD8 lymphocytes. Numerous studies have investigated anti-HIV specific CD8 lymphocyte responses but as these may be subverted by HIV immune evasion they do not reflect global CD8 lymphocyte function, and I will therefore focus on studies assessing general CD8 lymphocyte abilities, or CD8 lymphocyte responses to non-HIV pathogens.

Cytotoxic function. A number of investigators in the early years of HIV research noted decreased CTL responses in patients with AIDS. Thus in 1982, prior to the isolation of HIV, a paper was presented describing depressed anti-CMV CTL responses in subjects with AIDS (Frederick *et al.*, 1982), a finding replicated in a number of further studies (Rook *et al.*, 1983; Epstein *et al.*, 1985). Subsequently it was demonstrated that allogeneic CTL responses were also depressed, being present in only 4 of 19 subjects with AIDS or AIDS related complex (Sharma & Gupta, 1985). The lack of proliferative responses to alloantigen and mitogen demonstrated by Clerici *et al.*, (1989),(described in section 1.6.7.2), further support a profound loss of function of CD8 lymphocytes in the late stages of HIV disease.

The effector CTL response to CD3 stimulation in AIDS has been controversial, with some investigators finding loss of response (Gamberg *et al.*, 1999) while others found the response was maintained (Pantaleo *et al.*, 1990). The reason for these conflicting results is unclear, both Pantaleo and Gamberg used P815 mastocytoma cell lines as targets so there should not have been differential expression of pro apoptotic receptors.

Antigen stimulated IFN-γ production was also found to be defective in PBMC from subjects with AIDS. As IFN-γ can be produced both by CD4 and CD8 lymphocytes, the very low levels of production observed in advanced disease suggested dysfunction of both cell types (Murray *et al.*, 1988).

Once serological testing for HIV became available investigators were better equipped to study the asymptomatic phase of disease. Shearer *et al.*, (1986), assessed CTL activity of PBLs from asymptomatic and symptomatic HIV infected donors against influenza infected autologous targets and allogenic targets. PBLs were cultured for seven days with either influenza A or allogeneic cells, then the CTL response was tested by a 6 hour chromium release assay. Of 40 HTLV-III (later renamed HIV, see section 1.1) seronegative high risk individuals (largely homosexual men) all showed positive CTL responses to influenza and alloantigen, of 14 asymptomatic seropositive subjects 8 had strong responses to both stimuli but 6 responded only to alloantigen, while of 25 subjects with advanced disease, 2 responded to both stimuli, 11 responded to alloantigen only and 10 responded to

neither. Longitudinal study of a single subject confirmed progressive loss of response over time first to influenza then to alloantigen. In keeping with previous observations (Rook *et al.*, 1983), co-culture with IL-2 could rescue the anti-influenza response, and in healthy subjects with a good response to both antigens, the anti influenza response but not the anti-alloantigen response could be abrogated by depletion of CD4 lymphocytes. The authors conclude that HTLV-III infection results in progressive defects in CTL function that are initially consistent with lack of CD4 lymphocyte help, but in later stages are indicative of a more profound failure (Shearer *et al.*, 1986).

Precursor CTL function. A number of studies confirmed deterioration of CTL function with disease progression, and demonstrated that the CTL function can be recovered by various stimuli (Gruters *et al.*, 1990; Lewis *et al.*, 1994; Gamberg *et al.*, 1999). Thus Gruters *et al.*, (1990), demonstrated that CTL response to immobilized CD3 was normal in asymptomatic HIV infected subjects, but decreased in subjects with ARC or AIDS, and that function was almost wholly recovered by CD28 costimulation or addition of IL-2. Again, Gamberg *et al.*, (1999), demonstrated complete loss of the CD3 induced CTL response in freshly isolated PBMCs from 10 of 11 subjects with AIDS, but normal responses when PBMCs were first stimulated in an IL-2 containing medium.

Proliferation. Abnormalities in CD8 lymphocyte proliferation in HIV infected subjects have also been noted, including impaired response to CD3 stimulation in asymptomatics (Miedema *et al.*, 1988), and reduced response to IL-2 in subjects with high viral loads (Kryworuchko *et al.*, 2004). Use of a cell free CD3 stimulation methodology confirmed that the defect was in the responding T cells, not antigen presenting cells.

Anti-EBV CD8 lymphocytes. The relationship between HIV infection and EBV specific CD8 lymphocyte function has been extensively researched and has benefited from a number of longitudinal studies using prospectively cryopreserved PBMCs from untreated HIV infected subjects enrolled in the Amsterdam cohort.

A study in the 1980s indicated loss of anti-EBV CTL activity in HIV infected subjects. The level of activity correlated with CD4 lymphocyte count, and in early disease the defect could be reversed with IL-2 (Blumberg *et al.*, 1987). Subsequent studies have shown that numbers of functional anti-EBV precursor CTLs, are generally well preserved in HIV infected subjects, but drop in subjects developing EBV related tumors. Thus, in a cross sectional study using limiting dilution analysis with chromium release cytotoxicity assays, (a method which quantifies a subset of antigen specific CTL precursors that are able to proliferate and remain cytotoxic after prolonged stimulation), Carmichael *et al.,* (1993), found no difference in frequency of anti-EBV CTL precursors between HIV infected subjects, (including 5 with AIDS), and uninfected controls. In a detailed longitudinal study, (Kersten *et al.*, 1997). used similar methodology to show that anti-EBV CTL precursor frequencies remained stable over 5 to 8 years of follow up both in two long term non-progressors and two subjects who progressed slowly to opportunistic infection. In contrast, significant decline in EBV CTL precursor frequency was noted in two subjects who progressed rapidly to AIDS and in two subjects who developed EBV related tumors. In the subjects who developed EBV associated tumors the decline in anti-EBV CTL precursor frequencies preceded a rise in EBV load, suggesting that poor CD8 function plays a role in the loss of control of this virus. Two subsequent studies from the same group used tetramers to quantify circulating EBV specific CD8 lymphocytes, and found numbers to be normal throughout the course of HIV infection.

While numbers of EBV specific cells appear stable in HIV infection, there is increasing evidence that their function may be progressively compromised. In a cross sectional study of IFN-γ secretion in response to EBV peptides, the intensity of the response, as measured by enzyme linked immunospot (elispot) assay, correlated with CD4 lymphocyte count (suggesting deterioration in function with advancing HIV disease). In six subjects responses were assessed longitudinally, IFN-γ secretion was maintained over time in two subjects with stable CD4 lymphocyte counts and in two patients with declining CD4 lymphocyte counts. In the remaining two subjects the intensity of response declined with disease progression (Dalod *et al.*,

1999). A subsequent longitudinal study of 11 HIV infected subjects used the same assay to assess the relationship between anti-EBV CD8 lymphocyte function and the development of EBV related tumors. PBMCs isolated from HIV infected subjects within a few years of seroconversion showed decreased EBV specific IFN-γ secretion compared to PBMCs from uninfected controls. In HIV infected subjects that progressed to EBV associated tumors the IFN-γ production further deteriorated while in the remaining subjects there was no change in function with time (Van Baarle *et al.*, 2001).

Maturation of EBV specific CD8 lymphocytesin HIV infection has been assessed in terms of CD27 and perforin expression. Jansen *et al.*, 2004, found that as disease progressed EBV specific CD8 lymphocytes became more differentiated with a higher proportion expressing perforin and displaying the CD27- effector phenotype. Interestingly this progressive differentiation is absent in HIV infected subjects who progress to EBV associated tumors (Van Baarle *et al.*, 2002a).

Anti-HBV CD8 lymphocyte function. In a clear case controlled study (Lascar *et al.*, 2005), determined the effect of HIV infection on the CD8 lymphocyte anti hepatitis B virus (HBV) response in individuals who had recovered from HBV infection. The proportion of CD8 lymphocytes able to secrete IFN-γ in response to various hepatitis B virus peptides was assessed by intracellular staining in 16 HIV infected subjects at various stages of disease progression, compared with 14 HIV –ve controls. There was a marked decrease in response in the HIV infected compared to the control group, with only one of 16 HIV infected subjects having a detectable response compared to 9 of 14 controls. In two of four subjects studied longitudinally anti HBV responses recovered with reconstitution of CD4 lymphocytes following introduction of HAART.

Reconstitution with HAART. In addition to assessment of CD8 lymphocyte response during the natural history of disease there has been extensive investigation of recovery of immune function with therapy. (Hsieh *et al.*, 2000; Havlir *et al.*, 2000). A longitudinal study of the effect of HAART on EBV specific CD8 lymphocytes

demonstrated significant recovery of IFN-γ production that shadowed the rise in CD4 lymphocyte count (Kostense *et al.*, 2002a). Recovery of immune function is particularly important where the clinical question of withdrawal of long term prophylactic medication is raised, and large scale clinical trials have demonstrated that immune recovery sufficient to allow discontinuation of prophylaxis does occur (Ledergerber *et al.*, 2001). In some cases recovery of CTL response can be excessive, leading to immune restoration disease, (reviewed in (French *et al.*, 2004). Interestingly HIV specific CMV responses as measured by IFN-γ elispot assay increased initially with HAART but then declined. The decline could not be explained in terms of viral load or T cell counts. (Keane *et al.*, 2004).

Conclusion. Taken together, the above studies demonstrate that CD8 lymphocyte effector function deteriorates progressively during the course of HIV infection despite maintenance of normal numbers of antigen specific cells. Early in infection antigen specific IFN-γ production declines, while CTL function is largely maintained. As disease progresses IFN-γ production declines further, and circulating or effector CTL function is lost, while general precursor CTL function is maintained. Changes in CTL function with disease progression appear to vary for different pathogens and between different subjects. Thus EBV specific precursor CTL function is maintained in the majority of subjects into the advanced stages of disease, but in a subset of patients EBV specific CD8 lymphocytes lose the ability to mature to effector status, precursor CTL function and IFN-γ production is lost and the patients suffer increasing EBV loads and EBV related malignancy. CD8 lymphocytes specific for other pathogens appear to behave differently, thus the vast majority of subjects with AIDS have no detectable precursor CTL response to influenza, and the CTL response to TB appears to deteriorate even earlier, during asymptomatic disease. Elucidation of the mechanisms behind these varying responses is one of the major current challenges in HIV research

1.6.7.5 **Mechanism of CD8 lymphocytes dysfunction in HIV infection**

CD4 lymphocyte help. It is likely that the lack of CD4 lymphocyte help is a major reason behind poor CTL function in HIV (Kalams & Walker, 1998; Altfeld &

Rosenberg, 2000). CD4 lymphocytes secrete IL-2 which is vital for CD8 lymphocyte survival, and play an integral part in the activation of naïve CD8 lymphocytes. Thus in the CD4 lymphocyte depleted mouse, CD8 lymphocytes remain but do not function (Cardin *et al.*, 1996). The importance of CD4 lymphocyte help is supported by the recovery of CTL response *in vitro* by the addition of IL-2, and by the correlation between CD8 lymphocyte function and CD4 lymphocyte count (Dalod *et al.*, 1996; Kostense *et al.*, 2002a), but diminished production of IL-2 or other cytokines important in CD4 lymphocyte help has not been detected (Kaech *et al.*, 2002). While lack of CD4 lymphocyte help cannot directly explain the loss of CD4 lymphocyte independent functions such as the CTL response to alloantigen, it is possible that prolonged lack of CD4 lymphocyte help could contribute to lack of CD8 lymphocyte maturation or cause a state of anergy resulting in global hyporesponsiveness.

Direct and indirect cytotoxicity. Both direct and indirect cytotoxicity of HIV, described in section 1.3.4, are of relevance to CD8 lymphocyte function. Direct toxicity has been reported in HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes *in vitro.* Toxic effects included downmodulation of cell surface CD4 and CD8 expression, deregulation of IFN-γ and FASligand expression and decreased effector function (Kitchen *et al.*, 2004; Stove *et al.*, 2005). In addition CD4 independent HIV variants isolated from HIV infected subjects showed significant cytopathic effects on infected CD8 lymphocytes including syncytia formation (Zerhouni *et al.*, 2004). Some of the toxic effects of secreted viral proteins have been shown to affect CD8 as well as CD4 lymphocytes, for example vpr induces apoptosis of CD8 lymphocytes (Okada *et al.*, 1998).

Subset imbalance. It is likely that a number of abnormalities reported in unfractionated PBMC or CD8 lymphocyte populations from HIV infected subjects will reflect differences in the relative proportions of cells at different stages of maturation. Lewis *et al.*, 1994, found that HIV infected subjects have a significant expansion of the terminally differentiated CD57+ subset of CD8 lymphocytes. They demonstrated that these cells have markedly reduced proliferation in comparison to

their CD57- counterparts, regardless of HIV status. They therefore argue that the increased contribution of this subset to the overall population may explain the observed decreased proliferation of unfractionated CD8 lymphocyte populations.

Dysmaturation. While there is an overall expansion of effector cells in HIV infected subjects compared to healthy controls, within populations of antigen specific CD8 lymphocytes there may be a failure to differentiate to effector status (as described in section 1.6.7.4 for EBV specific CTLs). Currently it is unclear whether the lack of mature cells is due to a block in maturation, such as could be caused by HIV accessory proteins, or due to premature death of mature effector cells. Premature death could result from HIV infection of CD8 lymphocytes during activation, or from HIV induced apoptosis which preferentially effects preterminally differentiated cells (Mueller *et al.*, 2001).

Anergy. While anergy has been more extensively studied in CD4 lymphocyes, the defects in antigen presentation described in section 1.6.7.3, could equally lead to the state of reduced responsiveness observed in CD8 lymphocytes (reviewed in (Lieberman *et al.*, 2001). In addition, downregulation of cell surface CD8 expression could impair CD8 lymphocyte activation (Stove *et al.*, 2005).

Impaired signaling. (Kryworuchko *et al.*, 2004), showed that CD8 lymphocytes isolated from HIV infected subjects with high viral loads had impaired proliferative response to IL-2. The impaired proliferation was found to correlate with impaired activation of Jak-3, part of the Jak/stat signaling cascade. The mechanism by which HIV exerts this effect is unknown.

Natural killer cell inhibitory receptors. Natural killer cell inhibitory receptors (NKRs) are upregulated on CD8 lymphocytes in HIV infected subjects (Galiani *et al.*, 1999; De Maria *et al.*, 2000). These receptors are known to inhibit cytotoxicity and IFN-γ production (Huard & Karlsson, 2000) and in HIV infected donor samples blocking NKR with specific antibodies augmented HIV specific cytotoxicity (Demaria *et al.*, 1997).

1.6.8 Summary of section 1.6

PHI is characterized by massive HIV infection of memory CD4 lymphocytes within mucosae, lymph nodes and blood, associated with extensive apoptosis of effector CD4 lymphocytes such that these are effectively wiped out from mucosal sites. During the asymptomatic phase of chronic infection there is a steady decline in the numbers of both memory and naïve CD4 lymphocytes, with naïve cells declining more rapidly, and a decline in naïve CD8 lymphocytes. The cause of this decline is unclear but is likely to involve ongoing high turnover of antigen experienced CD4 and CD8 lymphocytes due to infection, immune activation or HIV induced apoptosis, together with difficulty in regeneration due to thymic involution and loss of lymph node architecture.

The extent to which the function of the remaining T lymphocytes is impaired in asymptomatic disease is unclear. Certainly there are deficiencies in proliferation and cytotoxic responses to some pathogens *in vitro*, while a number of secretory functions are maintained. The lack of proliferation and killing may be due to true impaired function *in vivo* or intolerance of culture conditions. Likely causes of impaired CD8 lymphocyte function include lack of CD4 lymphocyte help due to decreased numbers of effector CD4 lymphocytes, inhibitory effects of circulating HIV proteins, abnormal antigen presentation and changes in the cytokine environment. As disease progresses the lack of responses become incontroversial.

1.7 Importance of CD8 lymphocytes in controlling HIV infection

There is considerable evidence that CD8 lymphocytes are vital in the control of HIV replication both in primary and chronic HIV infection. HIV specific CD8 lymphocytes become detectable early during acute HIV infection and remain throughout the chronic phase (Moss *et al.*, 1995; Ogg *et al.*, 1998). *In vitro* these lymphocytes are able to kill HIV infected cells (though as discussed earlier this may require addition of IL-2 or overnight culture) (Yang *et al.*, 1996; Mueller *et al.*, 2001), and to inhibit HIV replication through an unidentified factor, CD8+ cell antiviral factor (CAF) (Walker *et al.*, 1986). Evidence that CD8 lymphocytes are

active against HIV *in vivo* is provided by the appearance of CTL escape mutants during acute and chronic infection (Borrow *et al.*, 1997).

Both correlative data and animal experiments indicate that CTL activity slows disease progression. Thus certain HLA-1 types (eg. HLA-B35), as well as homozygosity at the HLA-1 loci, are associated with rapid progression, while others (HLA B27 and HLA B57) are protective (Kaslow *et al.*, 1996b). In primary infection, the appearance of anti HIV CTLs is temporally associated with control of viral replication (Koup *et al.*, 1994), and increased diversity of the primary CTL response correlates with slow disease progression (Pantaleo *et al.*, 1997). In chronic infection, while the data is split, there does not appear to be a significant link between disease progression and the size or diversity of the cytotoxic response (Harrer *et al.*, 1996; Migueles & Connors, 2001), but slow progression has been found to correlate with enhanced CD8 lymphocyte proliferative responses (Migueles *et al.*, 2002).

Direct experimental evidence comes from SIV infected rhesus monkeys where deletion of the CTL response with anti CD8 antibodies causes failure of viral control both in acute and chronic infection (Schmitz *et al.*, 1999; Jin *et al.*, 1999). Finally, rhesus monkeys that are vaccinated to induce a CTL response and are then infected with SIV or SHIV have a more benign course than unvaccinated animals (Seth *et al.*, 2000).

1.8 Failure of the anti-HIV T lymphocyte response.

Despite evidence of significant T lymphocyte pressure, the early anti-HIV response fails and HIV continues to replicate, with an estimated 10 billion HIV virions produced daily in asymptomatic untreated individuals (Wei *et al.*, 1995; Ho *et al.*, 1995). The degree to which the anti-HIV CTL response is ineffectual was demonstrated by the superinfection of a subject, despite the presence of CTL responses against 8 epitopes present in the superinfecting virus (Altfeld *et al.*, 2002). The cause of the anti-HIV CTL failure has been extensively reviewed (McMichael & Rowland-Jones, 2001; Lieberman *et al.*, 2001; Piguet & Trono, 2001; Petrovas *et al.*, 2004), and is due to a combination of viral strategies to avoid recognition such as

mutation and down-modulation of cellular MHC expression, and defects in T lymphocyte function. Here the viral strategies for immune evasion are introduced, then the evidence for HIV specific T lymphocyte dysfunction is considered.

1.8.1 HIV immune evasion

Failure of CD8 lymphocytes to control HIV replication *in vivo* can be partly explained by viral strategies to avoid CTL recognition, (reviewed in (Altman & Feinberg, 2004). Thus the lack of fidelity of the viral reverse transcriptase and the viral tolerence for sequence change results in production of millions of new HIV variants each day. In the SIV model there is clear evidence of the rapid emergence of CTL escape mutants, associated with a decreased CTL viral control (Schmitz *et al.*, 1999), and similar findings have been reported for HIV. In addition HIV avoids recognition by downmodulation of MHC class I on infected cells, a strategy it shares with a number of other viruses. This downregulation is mediated by the HIV protein nef which diverts MHC I molecules away from the cell surface into the trans-Golgi network (Schwartz *et al.*, 1996).

1.8.2 Anti-HIV CD4 Lymphocyte response

There is good evidence that HIV specific CD4 lymphocytes are present in the majority of HIV infected subjects during the asymptomatic phase of infection. They are able to secrete cytokines (Pitcher *et al.*, 1999; Mathew *et al.*, 1999) but do not proliferate *in vitro* (Musey *et al.*, 1999; Rosenberg *et al.*, 1999). McNeil *et al.*, 2001, demonstrated that proliferation of HIV specific CD4 lymphocytes is actively inhibited by high levels of HIV viraemia, and can be rescued *in vitro* by CD28 costimulation. A similar pattern of diminished proliferative response during viraemia is seen in HBV (Boni *et al.*, 1998), measles (Whittle *et al.*, 1978), CMV (Carney & Hirsch, 1981) and dengue infection (Mathew *et al.*, 1999), and may reflect an anergic response to persisting high levels of antigen. Alternatively the lack of proliferation *in vitro* may reflect intolerance of culture conditions.

1.8.3 Anti-HIV CD8 lymphocyte response

1.8.3.1 **Size and breadth of response**

The acute anti HIV CTL response is large involving up to 10% of circulating CD8 lymphocytes (Koup *et al.*, 1994; Borrow *et al.*, 1994), dropping to 1 – 2% during chronic infecton (Altman *et al.*, 1996). Despite the identification of more than 180 optimal CTL epitopes spread throughout the HIV protein, the CTL response is often restricted to a few dominant epitopes (Lieberman *et al.*, 1997), a pattern that is also seen for other viral infections (Callan *et al.*, 1996). This narrow focus may promote immune escape, and assessment of CTL responses in a large cohort of 148 subjects showed that the breadth of response (ie the number of HIV epitopes which elicited a CTL response) was correlated with immune control of HIV replication and disease progression. In a related issue, it has been suggested that the large CD8 lymphocyte clonal expansions which occur in primary HIV infection could lead to clonal deletion as described in lymphocytic choriomeningitis virus (LCMV) infection of mice, resulting in restriction of the TCR repertoire (Moskophidis *et al.*, 1993). However other studies have found normal TCR diversity in chronic HIV disease, and where diversity has appeared restricted, reconstitution of a more diverse repertoire occurs following antiviral treatment (Boyer *et al.*, 1993; Gamberg *et al.*, 1999). Thus while the breadth of response is clearly important, the determinants of a broad or narrow response remain obscure.

1.8.3.2 **Function of anti-HIV CD8 lymphocytes**

The function of HIV specific CD8 lymphocytes has been assessed by comparison with CD8 lymphocytes specific to other antigens, and by comparison of function in rapid progressors against that in non-progressors. Interpretation of the significance of observations requires care. Differences noted using the former strategy may reflect defective function, or, as discussed later, may reflect a normal response tailored to a given pathogen. Differences in CD8 lymphocyte function noted between progressors and non-progressors may be causative, or could be the consequence of a less virulent viral strain or lower viraemia.

In the asymptomatic phase of HIV infection there is good evidence that HIV specific CD8 lymphocytes differ from CD8 lymphocytes specific for other chronic viral infections in terms of maturation, perforin expression and susceptibility to apoptosis. Some investigators also report defective cytotoxic function. The maturation of HIV specific CD8 lymphocytes appears to be truncated such that the majority of HIV specific CD8 lymphocytes have a CD45RO+CD27+CD28- phenotype classic of memory function with relatively few expressing the CD45RO+CD27-CD28- phenotype indicative of full differentiation to effector status (Ogg *et al.*, 1999; Roos *et al.*, 2000; Appay *et al.*, 2000). Similar findings were reported by Champagne *et al.*, 2001, using CD45RA and CCR7 as alternative markers of effector and memory phenotype, and by Mueller *et al.*, 2001, using CD45RA and CD62L. The cause of the skewed maturation is unknown, but possibilities include rapid loss of terminally differentiated cells (potentially caused by apoptosis), lack of CD4 lymphocyte help, or high dose antigen induced tolerance.

HIV specific CD8 lymphocytes have also been noted to be perforin deficient (Appay *et al.*, 2000), and this deficiency has been linked to an immature phenotype. Others find no difference in perforin expression between CMV-, EBV- or HIV specific CD8 lymphocytes, and suggest that low perforin expression is the norm in chronic viral infections (Zhang *et al.*, 2003). Lymphoid tissue from subjects with primary HIV disease display a relative lack of perforin when compared to primary EBV infection, suggesting that the lack of perforin is not a response to chronic disease, but is a response to something intrinsic to HIV itself (Andersson *et al.*, 2002).

The significance of the immature phenotype and lack of perforin expression is hotly debated. Some suggest it represents a failing of the immune system which contributes to loss of control of HIV replication (Van Baarle *et al.*, 2002b). Perforin is a vital component in the cytotoxic granules used to lyse target cells, so decreased perforin expression would logically lead to decreased effector function. In support, (Appay *et al.*, 2000), found that the lack of maturity was linked to defective cytolysis of HIV infected targets in a direct 4 hour chromium release assay. As discussed below, others refute that HIV specific CD8 lymphocytes have deficient cytotoxic function. Interestingly, in HIV infected subjects who lose control of EBV

replication, EBV specific CD8 lymphocytes also display an immature phenotype (Van Baarle *et al.*, 2002c, see section 1.6.7.4).

Others argue that CD8 lymphocytes in asymptomatic HIV infection are essentially 'fully functional' and the immature phenotype reflects an appropriate immune response to HIV (Appay & Rowland-Jones, 2002; Gamadia *et al.*, 2002; Zhang *et al.*, 2003). Preserved function of HIV specific CD8 lymphocytes is supported by studies showing normal ability to secrete antiviral cytokines such as IFN-γ, TNF-α and macrophage inflammatory protein-1β (MIP-1β) (Dalod *et al.*, 1999; Appay *et al.*, 2000), and by studies demonstrating the anti-HIV immune pressure applied by CD8 lymphocytes *in vivo* which continues throughout the asymptomatic stage of HIV disease (Kelleher *et al.*, 2001).

An increased susceptibility to apoptosis was noted by Mueller *et al.*, 2001, who found HIV specific CD8 lymphocytes to be 3 times more sensitive to Fas-induced apoptosis when compared to CMV specific CD8 lymphocytes from the same donors. This increased sensitivity was most marked in the preterminally differentiated (memory) cells, leading the authors to suggest that the lack of fully differentiated CD8 lymphocytes is a result of increased apoptosis just prior to terminal differentiation.

Some investigators report that the cytotoxic function of HIV specific CD8 lymphocytes is abnormal (Shankar *et al.*, 2000; Appay *et al.*, 2000) while others have found no such defect (Mueller *et al.*, 2001). It has been suggested that the experiments showing normal cytotoxic function were confounded by use of overnight culture, or culture in IL-2 containing media prior to the cytotoxicity assays, both of which could mask a functional deficit (Walker *et al.*, 1987; Trimble & Lieberman, 1998; Lieberman *et al.*, 2001). However, this does not explain the observation of normal cytotoxicity in freshly isolated cells (Mueller *et al.*, 2001). Mueller *et al.*, 2001, argue that the discrepant findings may reflect the different targets used. Thus, in the studies showing impaired cytotoxicity EBV transformed B cells were used as targets, and, as these cells express Fas-L they are likely to induce apoptosis in the HIV specific CTLs and underestimate their cytotoxic ability.

Numerous studies have been performed comparing CD8 lymphocyte function in LTNP against that in rapid progressors in an attempt to elucidate correlates of non-progression (Kostense *et al.*, 2001; Migueles *et al.*, 2002; Van Baarle *et al.*, 2002a). Results have often been contradictory, for example some investigators found HIV specific CD8 lymphocyte of non-progressors to be more differentiated (Van Baarle *et al.*, 2002a), and have better IFN-γ responses to antigenic stimuli than those of rapid progressors (Kostense *et al.*, 2001), while others have found no difference in terms of IFN-γ secretion (Migueles *et al.*, 2002) or differentiation (Appay & Rowland-Jones, 2002). One recent report in which the non-progressor group had maintained undetectable serum viral loads over at least 5 years of untreated infection, found no difference between the two groups in the number or diversity of HIV specific CD8 lymphocytes and their response to autologous HIV infected CD4 lymphocytes was similar in terms of activation and IFN-γ secretion, but proliferation and perforin expression was much more marked in the LTNPs (Migueles *et al.*, 2002).

Inefficient trafficking of HIV specific CD8 lymphocytes to lymph tissue, where the majority of infected cells are sited, has also been proposed as a factor in their failure to control HIV replication (Chen *et al.*, 2001). Inefficient trafficking to lymph nodes is not unexpected as priming classically leads to downregulation of lymph node homing receptors.

While there is controversy regarding the function of CD8 lymphocytes in the asymptomatic stage of chronic infection, it is generally agreed that in advanced stages HIV specific CD8 function is impaired as evidenced by reduced cytolytic activity (Pantaleo *et al.*, 1990) impaired cytokine production (Kostense *et al.*, 2002b) and reduced CD3ζ expression (Trimble & Lieberman, 1998).

As noted in section 1.6.7.4 lack of CD4 help is a likely cause of generalized CD8 lymphocyte dysfunction in HIV disease. Of relevance to anti-HIV CD8 lymphocyte function, the CD4 lymphocyte help required for primary CD8 lymphocyte activation is exclusively provided by a CD4 lymphocyte of the same specificity, so primary activation of an HIV specific CD8 lymphocyte often requires the participation of an HIV specific CD4 lymphocyte. Thus, the early deterioration of the anti-HIV CD4 lymphocyte response (described in section 1.6.7.2) could contribute to the failure of

the anti-HIV CTL response in asymptomatic disease. A role for CD4 lymphocytes is supported by the recovery of the anti-HIV CTL response *in vitro* by addition of IL-2, and by the correlation between the strength of the anti-HIV CD4 lymphocyte response and control of viraemia (Rosenberg *et al.*, 1997).

1.8.3.3 **Summary of section 1.8**

In summary, an anti-HIV T lymphocyte response is initiated early in infection but is unable to control infection due immune evasion strategies and massive loss of the CD4 lymphocyte effector arm. After a few weeks of viraemia the function of HIV specific lymphocytes declines. Thus in asymptomatic disease HIV specific CD4 lymphocytes are able to secrete IFN-γ but are deficient in proliferation, while anti-HIV CD8 lymphocytes do not mature to effector status and may have reduced cytotoxicity. These abnormalities occur at an earlier stage than similar abnormalities in T lymphocytes specific for other antigens, and therefore must be caused by mechanisms acting preferentially on the HIV-specific cells. Likely mechanisms include HIV infection, which occurs more readily in HIV-specific CD4 lymphocytes, anergy induced by the very high levels of antigen, or antigen processing defects. In addition, the proximity of HIV-specific T lymphocytes to infected cells during activation and effector functions may lead to increased risk from the cytotoxic effects of secreted HIV proteins.

1.9 HIV Reservoirs.

The persistence of HIV is largely due to its ability to establish non-cytopathic infection of long-lived cells that are relatively hidden from immune surveillance, a topic extensively reviewed by Pierson *et al.,* (Pierson *et al.*, 2000). Populations of these cells form viral reservoirs that are unaffected by antiviral therapy, allowing recurrence of infection after years of combination therapy. In addition these reservoirs can harbor virus generated at any time during the history of infection of an individual, including virus with drug resistant mutations which may have developed during therapeutic regimens that have since been superceded. This historical record

means that patients tend to have only a single opportunity with any given antiviral regimen, limiting the choices available once regimens need to be altered. This topic is of particular interest as at least some HIV infected CD8 lymphocytes are likely to be longlived and therefore to contribute to these reservoirs.

The existence of viral reservoirs was revealed by analysis of the decay dynamics of plasma viral load after initiation of combination antiviral therapy. Using methods for viral load measurement sensitive to 50 copies per ml, two distinct phases were observed with half lives of 1-2 days and 14 days, corresponding to virus production by activated CD4 lymphocytes and macrophages (Perelson *et al.*, 1997). Extrapolation of the second stage lead to the prediction that three years of antiviral therapy would be sufficient to eradicate the virus. This has proved not to be the case and two further phases of decay have been reported. Phase three has a half life of 6 months and is thought to reflect virus produced by resting (G_1) T cells and possibly dendritic cells. The forth phase has a half life of 43 months, and is due to infection of quiescent (G_0) T cells (Chun *et al.*, 1995; Finzi *et al.*, 1999). This quiescent lymphocyte reservoir was demonstrated by viral production following *ex vivo* activation of lymphocytes isolated from HIV infected individuals on HAART (Wong *et al.*, 1997; Finzi *et al.*, 1997). Thus there is no longer an expectation that HIV can be eradicated by current antiviral therapy alone, and a thorough understanding of the dynamics of the various long-lived cell types infected has become paramount.

The mechanism of infection of quiescent cells is intriguing as activated CD4 lymphocytes do not generally revert to a quiescent state (Stevenson, 2003) and truly quiescent (G_0) lymphocytes are refractory to infection (Zack *et al.*, 1990). Recent evidence indicates that subtle stimulatory signals, which can be produced by HIV infected macrophages in a nef dependent manner, may move G_0 lymphocytes into G_1 without inducing full activation (Swingler *et al.*, 2003). Such cells are permissive to infection, and may revert back to a truly quiescent state.

Macrophages also serve as an important reservoir for HIV, with a number of factors enhancing the longevity of infected cells. Firstly, non-dividing macrophages are permissive to HIV infection (Weinberg *et al.*, 1991). Retroviruses usually require cell division to allow cDNA to enter the nucleus, but HIV nuclear targeting signals

guide the reverse transcription complex through nuclear pores thus allowing infection in the absence of cell division (Bukrinsky *et al.*, 1993). Secondly, macrophages are relatively resistant to the cytopathic effects of HIV. Finally the assembly of HIV virions in vesicles rather than on the plasma membrane renders the macrophages less visible to HIV specifc CTLs (Orenstein *et al.*, 1988).

The CNS is also a potentially important reservoir as it is an immunologically privileged site and penetration of antiretrovirals such as protease inhibitors is suboptimal (Hughes *et al.*, 1997b). HIV enters the CNS soon after seroconversion (Davis *et al.*, 1992; Bell *et al.*, 1993), and infection of macrophages and microglial cells in the CNS have been demonstrated (Takahashi *et al.*, 1996; Hughes *et al.*, 1997a). Whether these cells act as a source of virus to reseed the general circulation after therapy is not known.

1.10 Evidence of HIV infection of CD8 lymphocytes.

It is well established that CD8 lymphocytes are susceptible to HIV infection *in vitro* (Flamand *et al.*, 1998; Yang *et al.*, 1998; Kitchen *et al.*, 1998; Zhang *et al.*, 2001; Zloza *et al.*, 2003); (De Maria *et al.*, 1991) and an increasing number of investigators have demonstrated HIV infection of CD8 lymphocytes *ex vivo* (Semenzato *et al.*, 1995; Livingstone *et al.*, 1996; Flamand *et al.*, 1998; Semenzato *et al.*, 1998; McBreen *et al.*, 2001; Imlach *et al.*, 2001; Brenchley *et al.*, 2004a).

Infection of CD8 lymphocytes *in vitro* was first reported in the 1980s, initially in HTLV-1 transformed CD8 lymphocyte clones, and then in cultured PBMCs isolated from HIV infected subjects. Efficient infection of CD8 lymphocytes required co-culture with HIV infected CD4 lymphocytes and resulted in productive infection (De Maria *et al.*, 1991). More recently a number of investigators have demonstrated HIV infection of activated CD8 lymphocytes without the presence of CD4 lymphocytes. These studies have shown unequivocally that HIV can enter CD8 lymphocytes, and that these cells can support viral replication, but the question as to whether this occurs to any significant degree in HIV infected people has remained controversial (Agostini & Semenzato, 2002).

In the mid 1990s two papers reported low frequencies of HIV infection of CD8 lymphocytes in humans (Semenzato *et al.*, 1995; Livingstone *et al.*, 1996), and a third paper described significantly higher levels of CD8 lymphocyte infection in SIV infected macaques (Dean *et al.*, 1996). Semenzato *et al.* (1995) demonstrated HIV provirus in CD8 lymphocytes isolated from the lung of HIV infected subjects, with infection rates ranging from 70 to 3960 proviral copies per 10^6 CD8 lymphocytes. Livingstone *et al.*, (1996) reported slightly lower frequencies of infection in CD8 lymphocytes isolated from blood (ranging from less than 1 to 400 proviral copies per 10^6 CD8 lymphocytes). Subsequently these findings were repeated by a number of investigators (Flamand *et al.*, 1997; Semenzato *et al.*, 1998; McBreen *et al.*, 2001; Imlach *et al.*, 2001; Potter *et al.*, 2003; Brenchley *et al.*, 2004a), all reporting low frequencies of infection in blood derived CD8 lymphocytes. All these investigators used a similar methodology involving isolation of highly purified CD8 lymphocyte populations from HIV infected donors, extraction of DNA then quantification of HIV provirus per cell by PCR. Other investigators have been unable to detect HIV infection of CD8 lymphocytes recovered *in vivo* (Levy, 1994) and critics have argued that the provirus observed could be explained by CD4 lymphocyte or free virion contamination of the isolated CD8 lymphocyte populations (Levy, 1994; Agostini & Semenzato, 2002; Brenchley *et al.*, 2004a).

HIV RNA has been reported in CD8 lymphocytes from lung and blood, suggesting productive infection of these cells. The first report, published in 1995, was based on data from a single patient. HIV RNA was demonstrated by RT-PCR in CD8 lymphocytes isolated from lung of an HIV infected subject. The proviral load was 162 copies per 10^5 CD8 lymphocytes, and HIV RNA was detected in 10^4 cell equivalents of extracted RNA, suggesting that at least 1 in 16 HIV infected CD8 lymphocytes were productively infected. The purity is quoted as >99% CD8 lymphocytes with 'virtually no detectable residual CD4 lymphocytes', and given that CD4 lymphocytes represented only 1% of the cells in the unfractionated broncho-alveolar lavage, it is unlikely that this finding is due to contamination (Semenzato *et al.*, 1995). These findings were subsequently confirmed in four additional subjects (Semenzato *et al.*, 1998). A separate study, presented in poster format at the 9th Conference on Retroviruses and Opportunistic Infections, demonstrated HIV RNA in

blood derived CD8 lymphocytes from all of 20 HIV infected subjects investigated using ultrasensitive fluorescence *in situ* hybridisation. This work has not been formally published (Rubbert *et al.*, 2005).

1.11 Mechanism of HIV infection of CD8 lymphocytes

Both CD4 dependent and independent mechanisms have been proposed to explain HIV entry into CD8 lymphocytes.

1.11.1 CD4 dependent mechanisms

CD8 lymphocytes express CD4 and become vulnerable to CD4 dependent HIV entry at two stages in their life cycle, firstly during intrathymic development, and secondly following antigen specific activation. These two opportunities for infection of CD8 lymphocytes are considered in detail in section 4.1. Infection of CD8 lymphocytes with either HTLV-1 or HHV-6 induces cell surface CD4 expression providing a further opportunity for HIV infection in co-infected individuals (Lusso *et al.*, 1991; Macchi *et al.*, 1993). Seroprevalence for HTLV-1 is increasing worldwide, reaching 30% in some IVDU populations (de Araujo *et al.*, 1994; Brites *et al.*, 1997; Guimaraes *et al.*, 2001). Seroprevalence for HHV-6 is almost universal in most populations, but lymphocytes are infected at low frequency and HHV-6 was not found in HIV infected CD8 lymphocytes isolated from lung (Semenzato *et al.*, 1995). Thus, while co-infection does not account for all the CD8 lymphocyte infection observed *in vivo* it may play an important role in selected populations, and increased CD8 lymphocyte infection could explain the accelerated disease progression observed in HTLV-1 / HIV co-infected individuals (Schechter *et al.*, 1994; Brites *et al.*, 1998; Brites *et al.*, 2001).

1.11.2 CD4 independent mechanisms.

HIV can also infect CD8 lymphocytes via CD4 independent mechanisms including coreceptor mediated infection, trans-mediated infection and CD8 dependent or novel mechanisms.

In vitro interactions between gp120 and the coreceptors CXCR4 and CCR5 can initiate viral entry in the absence of CD4 binding (Hesselgesser *et al.*, 1997; Dumonceaux *et al.*, 1998; Bandres *et al.*, 1998). While these interactions occur relatively frequently for SIV (Potempa *et al.*, 1997; Edinger *et al.*, 1997) and HIV-2 (Endres *et al.*, 1996; Potempa *et al.*, 1997; Reeves & Schulz, 1997), it is generally considered that they are too inefficient to play a major role in HIV-1 (Meireles-de-Souza & Shattock, 2005).

Trans-mediated infection occurs where neighbouring cells, one expressing CD4, and the other expressing CCR5 or CXCR4, cooperate to permit virus cell fusion. This mechanism allows infection of CD8 lymphocytes when co-cultured with CD4 expressing cells and is dependent on direct cell contact (De Maria *et al.*, 1991; Speck *et al.*, 1999). A similar mechanism may be responsible for infection of HIV specific effector CD8 lymphocytes on interaction with HIV infected target cells (De Maria *et al.*, 1994), but no preferential infection of HIV specific CD8 lymphocytes has been demonstrated *in vivo* (Brenchley *et al.*, 2004a).

Co-infection with HTLV-1 may lead to HIV infection of CD8 lymphocytes through the production of progeny virus with altered cell fusion requirements. HIV able to infect CD8 lymphocytes in a CD4 independent manner were generated by this method *in vitro*, but whether this occurs to a significant degree *in vivo* is unknown (Lusso *et al.*, 1990).

Two HIV variants have been isolated that infect CD8 lymphocytes through attachment to the CD8 molecule (Saha *et al.*, 2001). These variants were isolated from immortalized HIV infected $CD8^{+}CD4^{-}$ T cells isolated from a single AIDS patient, and were shown to infect highly purified $CD8^{+}$ PBLs and a cell line engineered to express CD8. Infection was blocked by anti CD8 antibodies and surprisingly did not require either of the co-receptors CXCR4 or CCR5. These variants showed novel sequence variations in the env gene, and these changes were thought to be responsible for their unusual tropism.

Subsequently the same group isolated CD4 independent viruses from 7 of 12 randomly selected HIV infected subjects, leading the authors to suggest that these variants commonly circulate at low frequency within the HIV quasispecies.

Infection of CD8+ cells by these variants was not inhibited by either anti CD4 or anti CD8 antibody, and some of the variants were able to infect cell lines negative for all common HIV co-receptors suggesting a novel mechanism for cell entry (Zerhouni *et al.*, 2004). All the subjects from whom CD4 independent virus was isolated were infected with X4 or R5X4 viruses that usually appear late in HIV-1 disease, the authors therefore speculate that CD4 independent variants may evolve with disease progression. The CD4 independent variants had changes in the variable loops or transmembrane portion of the env gene when compared to CD4 tropic virus from the same subjects, but no consensus sequence for CD4 independence was found.

1.11.3 Phylogenetic data.

Phylogenetic relationships between virus isolated from CD8 lymphocytes and that isolated from other cell types or from plasma could provide insights into the mechanism of viral entry, the contribution of the infected cell type to the circulating viral pool and the longevity of the infected cells. For example, if the usual mode of entry to CD8 lymphocytes was through novel receptor use, one would expect variation in the env gene that would make CD8 tropic viruses cluster separately from the virus isolated from CD4 expressing cells. Distinct clusters could also be explained by distinct lifespans of the cell populations studied.

McBreen *et al.*, (2001), assessed the phylogenetic relationships between V3 hypervariable region sequences amplified by PCR and cloning from CD8 lymphocytes and CD4 lymphocytes. Of the six subjects studied CD8 lymphocyte sequences were monophyletic in a single patient. In this patient plasma sequences grouped with CD4 derived sequences and contained a positively charged amino acid at residues 306 and 320, a genotype indicative of CXCR4 virus, while the CD8 lymphocyte derived sequences displayed the CCR5 virus genotype. This suggests that, in this patient, either the HIV infected CD8 lymphocytes were longlived, having been infected at a time prior to the R5 to X4 switch, or that CD8 lymphocytes were more easily infected by R5 varients. In four other patients there was partial separation of CD4 and CD8 sequences with the last patient showing random mixing.

In a separate study Potter *et al.*, (2003), assessed drug resistance mutations in provirus amplified from various cell types isolated from 10 HIV infected patients receiving HAART. Overall the prevalence of resistance mutations was higher in HIV infecting CD4 lymphocytes and monocytes than it was in HIV from CD8 lymphocytes, though there was marked heterogeneity between individual patients. Thus, in five subjects virus from CD8 lymphocytes had fewer drug resistance mutations than CD4 lymphocyte or monocyte derived virus, while the remainder showed no difference. Relationships between CD8 lymphocyte derived and plasma derived virus were also assessed in 4 subjects, and again considerable inter-patient variability was noted. In two patients CD8 lymphocyte sequences were closely related to plasma sequences (suggesting either recent infection of the CD8 lymphocytes or productive infection of CD8 lymphocytes), but in the other two the plasma and CD8 lymphocyte derived sequences were segregated.

The interpretation of the phylogenetic data presented in these two papers is difficult due to inter-patient variability. The variability is likely to reflect different dynamics of infection of the different populations observed. Alternatively, some of the mixing of CD8 lymphocyte sequences into those derived from CD4 lymphocytes may have been due to contamination of CD8 lymphocyte cell populations with CD4 lymphocytes, and the appearance of distinct populations could have been secondary to cloning induced artefact.

1.11.4 Summary of HIV infection of CD8 lymphocytes

CD8 lymphocytes are susceptible to HIV infection under various culture conditions, and a number of investigators have found low frequency infection *ex vivo*. The mechanism of infection *in vivo* remains unclear, with conflicting data from the studies published to date.

1.12 Scope of thesis.

As discussed in section 1.10, there is now considerable evidence that HIV commonly infects CD8 lymphocytes *in vivo*, but general acceptance of this concept has been inhibited by concerns regarding the purity of the CD8 lymphocyte populations

studied. These concerns are addressed in chapter three, through development of a method for isolation and verification of highly purified populations of CD8 lymphocyte subsets. This process necessitated cell fixing and therefore a method for DNA extraction from fixed tissue was also developed. Finally in this chapter a method for real-time PCR quantification of HIV provirus is described, this proved to be unsuitable for sole use with samples where the frequency of infection was low but was useful as an initial step prior to limiting dilution analysis.

In chapter four the extent of HIV infection of CD8 lymphocyte populations isolated from the blood of 20 HIV infected subjects is addressed. To determine the likely route of infection, CD8 lymphocyte populations were subdivided on the basis of differentiation phenotype or CD4 co-expression. The data presented confirms the presence of HIV infection of CD8 lymphocytes *ex vivo* and demonstrates high levels of infection in $CD8^{bright}CD4^{dim}$ lymphocytes. This suggests that infection occurs following upregulation of CD4 expression during activation rather than during intrathymic development.

Given the potential role of $CD8^{bright}CD4^{dim}$ lymphocytes in HIV immunopathology, a clear understanding of their natural history and function is important. Current concepts are almost wholly derived from *in vitro* experiments, therefore in chapter 5 the differentiation and activation status of $CD8^{bright}CD4^{dim}$ lymphocytes *ex vivo* is addressed. To determine whether HIV infection had any impact on their numbers, differentiation or activation status, data is presented both for HIV infected subjects and healthy controls. Finally, in chapter 6, the relevance of HIV infection of CD8 lymphocytes to HIV immunopathology is discussed.

Chapter 2: General Methods.

2.1 Isolation of lymphocyte populations.

Highly purified populations of CD4 lymphocytes and CD8 lymphocytes subsets were isolated from all donated blood samples. As each sample contained insufficient CD8 lymphocytes to assess HIV infection of all the cell subsets of interest, the samples were divided into two groups. CD8 lymphocytes from group 1 samples were isolated on the basis of CD4 expression yielding populations of $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes, and those from group 2 were isolated on the basis of differentiation phenotype yielding antigen naïve, antigen experienced, memory and effector subsets. γδ-T lymphocytes were also isolated from ten subjects and monocytes from three. The populations were isolated using a three step protocol first PBMC isolation, then immunomagnetic enrichment of lymphocyte populations, and finally fluorescent activated cell sorting. The overall strategy is shown in fig 2.1.

2.1.1 General notes on lymphocyte isolation.

In order to minimize cell metabolism, cells were maintained at 4°C unless otherwise stated. To pellet cells the cell suspension was centrifuged at 250g for a maximum of ten minutes. When resuspending cells or mixing cells with reagents this was done by gentle pipetting up and down. To wash cells, the cells were pelleted, the supernatant removed either by pouring off or using a Pasteur pipette, the cells were resuspended in the wash buffer, then re-pelleted, the wash buffer removed and the cells resuspended as required. Cells were counted at various stages in the process using a haemocytometer under light microscopy, and viability was checked using trypan blue (SigmaAldrich).

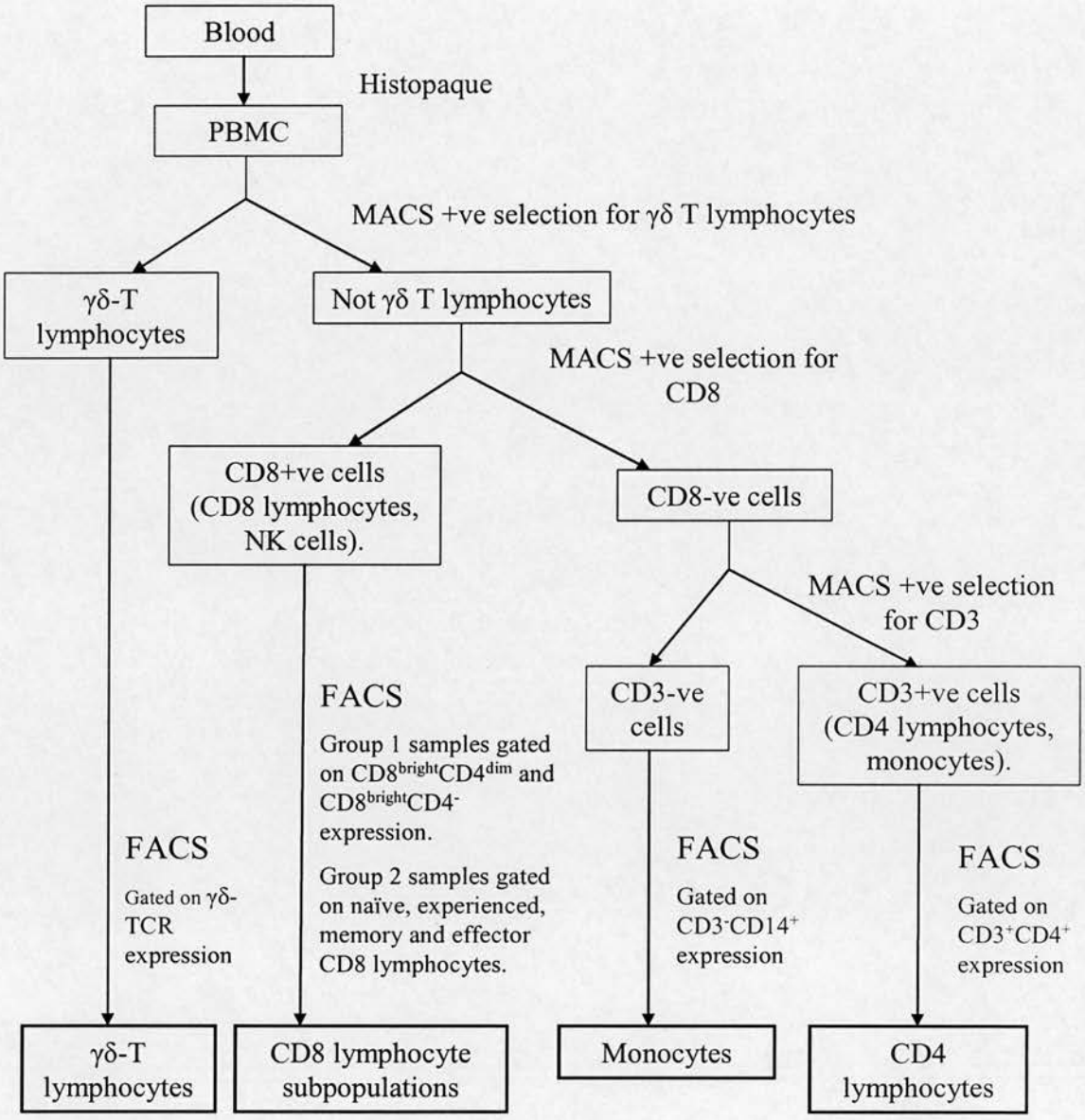


Figure 2-1. Schematic diagram of protocol for isolation of cell populations of interest. PBMC = peripheral blood mononuclear cells, MACS = immunomagnetic cell sorting, FACS = fluorescent activated cell sorting.

2.1.2 Isolation of PBMCs by density centrifugation.

Blood samples were taken into tubes containing ethylenediaminetetraacetic acid (EDTA, Sarstedt, Leicester, UK). Within 6 hours of sample donation, the blood was diluted 1:1 with phosphate buffered saline without calcium or magnesium (PBS,

Invitrogen, Paisley, UK), and 15ml of diluted blood was layered over 10ml of Histopaque in 25ml universal tubes (Sterilin, Staffordshire, UK) at room temperature (Sigma Diagnostics, St. Louis, USA). The tubes were centrifuged at 400g for 30mins with no brake in a Heraeus Christ centrifuge. The PBMCs were then removed from the interface using a Pasteur pipette, combined into a single universal tube, and washed twice in PBS. Total cells were counted then either stored overnight at 4°C in 3mls of 10% autologous serum in automax buffer (AMB, see section 2.8), or taken directly to immunomagnetic enrichment.

2.1.3 Immunomagnetic enrichment of T lymphocyte populations.

All immunomagnetic selection was performed using MACS reagents (Miltenyi Biotec Ltd (UK), Bisley, UK) and an autoMACS machine (Miltenyi Biotec Ltd.). Up to three steps were employed depending on the CD8 lymphocyte subsets to be isolated.

Step 1. Immunomagnetic depletion of γδ-T lymphocytes. PBMCs were pelleted and resuspended in 40μl AMB per 10^7 total cells. MACS γδ-hapten antibody (Miltenyi Biotec) was then added (10μl per 10^7 total cells), mixed, then incubated for 10 minutes. Cells were then washed in 20ml AMB, pelleted and resuspended in 90ml AMB per 10^7 total cells. MACS anti hapten FITC microbeads (Miltenyi Biotec) were added (10μl per 10^7 total cells), mixed, then incubated for 15 minutes. Cells were washed in 20ml AMB, resuspended in 1ml AMB and separated using program POSSEL on an autoMACS (Miltenyi Biotec). The cells obtained in each fraction were then counted, and the negative (unlabelled) fraction, depleted of γδ-T lymphocytes, was taken forward for CD8 lymphocyte isolation.

Step 2. Immunomagnetic enrichment of CD8 lymphocytes. The appropriate cells were pelleted and resuspended in 80μl AMB per 10^7 total cells. MACS CD8 microbeads (Miltenyi Biotec) were added (20μl per 10^7 total cells), mixed, then incubated for 10 minutes. Cells were washed in 20ml AMB, resuspended in 1ml AMB and separated using program POSSEL on an autoMACS (Miltenyi Biotec). The cells obtained in each fraction were then counted, and the positive (labelled)

fraction, enriched for CD8 expressing cells, was saved for FACS. The negative fraction was taken forward to step 3.

Step 3. Immunomagnetic enrichment of CD3 lymphocytes. The appropriate cells were pelleted and resuspended in 80µl AMB per 10^7 total cells. MACS CD4 microbeads (Miltenyi Biotec) were added (20µl per 10^7 total cells), mixed, then incubated for 10 minutes. Cells were washed in 20ml AMB, resuspended in 1ml AMB and separated using program POSSEL on an autoMACS (Miltenyi Biotec). The cells obtained in each fraction were then counted, and the positive (labelled) fraction, enriched for CD3 expressing cells, was used for isolation of CD4 lymphocytes and the negative fraction was used for isolation of monocytes.

2.1.4 Fluorescent Activated Cell Sorting.

Immunomagnetically enriched cells were stained with a combination of fluorescence conjugated monoclonal antibodies (Table 2-1) and sorted using a Vantage flow sorter (Becton Dickenson, Crawley, UK) to generate highly pure populations of CD8 lymphocytes, CD4 lymphocytes, γδ-T lymphoytes and monocytes (Figure 2-1). The sorter was calibrated daily using calibrite beads (Becton Dickenson), and was operated by a trained technician.

The phenotypes used to define the populations of interest are given (Table 2-2). To ensure that the population of $CD8^{bright}CD4^{dim}$ lymphocytes did not contain CD4 lymphocytes expressing CD8, the anti CD8 monoclonal employed was directed against the β chain of the CD8 molecule which is only present on true CD8 lymphocytes (Norment & Littman, 1988; Shiue *et al.*, 1988; Hori *et al.*, 1991).

Gates were drawn by eye for clear discrete populations, lymphocyte and monocyte forward and side scatter, naïve CD8 lymphocytes ($CD27^{high}CD45RA^{high}$), and $CD8^{bright}CD4^{dim}$ lymphocytes. The latter was positioned to maximise inclusion of all $CD8^{bright}$ cells with any CD4 expression and to avoid any $CD4^{bright}CD8^{-}$ cells. Gates for the memory and effector CD8 lymphocytes were positioned with the aid of

isotype controls. The position of gates for CD4 lymphocytes and CD8 lymphocyte subpopulations of a typical sample are shown, (Figure 2-2).

Cells were sorted into 3.5ml FACS tubes (Becton Dickinson), or 1.5ml microtubes (Eppendorf) depending on the number of cells expected. To avoid the electrically charged droplets sticking to the inside of the tubes, the inner surface was coated with sterile media (RPMI, invitrogen) prior to collection.

Cell population stained	**Populations to be isolated**	**Fluorescent conjugated monoclonal antibodies**
Cells enriched for γδ-T lymphocytes	γδ-T lymphocytes	γδ-TCR PE
Cells enriched for CD8 expression (Subjects AC14 – AC18)	$CD8^{bright}CD4^{dim}$ & $CD8^{bright}CD4^{-}$	CD8β chain PE CD4 Cy-chrome™
Cells enriched for CD8 expression (Subjects AC22-AC25)	$CD8^{bright}CD4^{dim}$ & $CD8^{bright}CD4^{-}$	CD8β chain PE Tubulin PC7 CD4 APC
Cells enriched for CD8 expression (subject AC26)	$CD8^{bright}CD4^{dim}$ & $CD8^{bright}CD4^{-}$	CD4 FITC CD8β chain PE Tubulin PC7
Cells enriched for CD8 expression	Antigen naïve, antigen experienced, memory and effector CD8 lymphocytes	CD27 FITC CD8β chain PE CD45RA Cy-chrome™ CD4 APC
Cells enriched for CD3 expression	CD4 lymphocytes	CD3 PE CD4 Cy-chrome™
Cells not expressing CD3 or CD8	Monocytes	CD14-PE CD3 cychrome

Table 2-1. Monoclonal antibody combinations used for FACS. The combination used for CD8 lymphocytes in group A was changed after the first five patients to allow exclusion of dead cells by costaining with tubulin PC7, and to avoid artefactual events mimicking CD4+ve cells (see section 4.3.1.2 , Figure 4-1). CD4 APC was subsequently dropped in favour of CD4 FITC due to better performance in the Vantage flow sorter. Details of clone and manufacturer given in table 2.3.

Lymphocyte subset	Phenotype
CD4 lymphocyte	$CD4^+ CD3^+$
Naïve CD8 lymphocyte	$CD8\beta^+ CD45RA^{high} CD27^{high}$
Antigen experienced CD8 lymphocyte	All $CD8\beta^+$ lymphocytes not falling into the naïve population.
Memory CD8 lymphocyte	$CD8\beta^+ CD45RA^{low} CD27^+$
Effector CD8 lymphocyte	$CD8\beta^+ CD45RA^+ CD27^-$
CD8 lymphocyte expressing CD4	$CD8\beta^{high} CD4^+$
CD8 lymphocyte not expressing CD4	$CD8\beta^+ CD4^-$
γδ-T lymphoytes	$\gamma\delta\text{-}TCR^+$
Monocytes	CD14+CD3-

Table 2-2. Phenotypes used to define cell populations isolated by FACS. All populations were also gated on appropriate forward and side scatter characteristics.

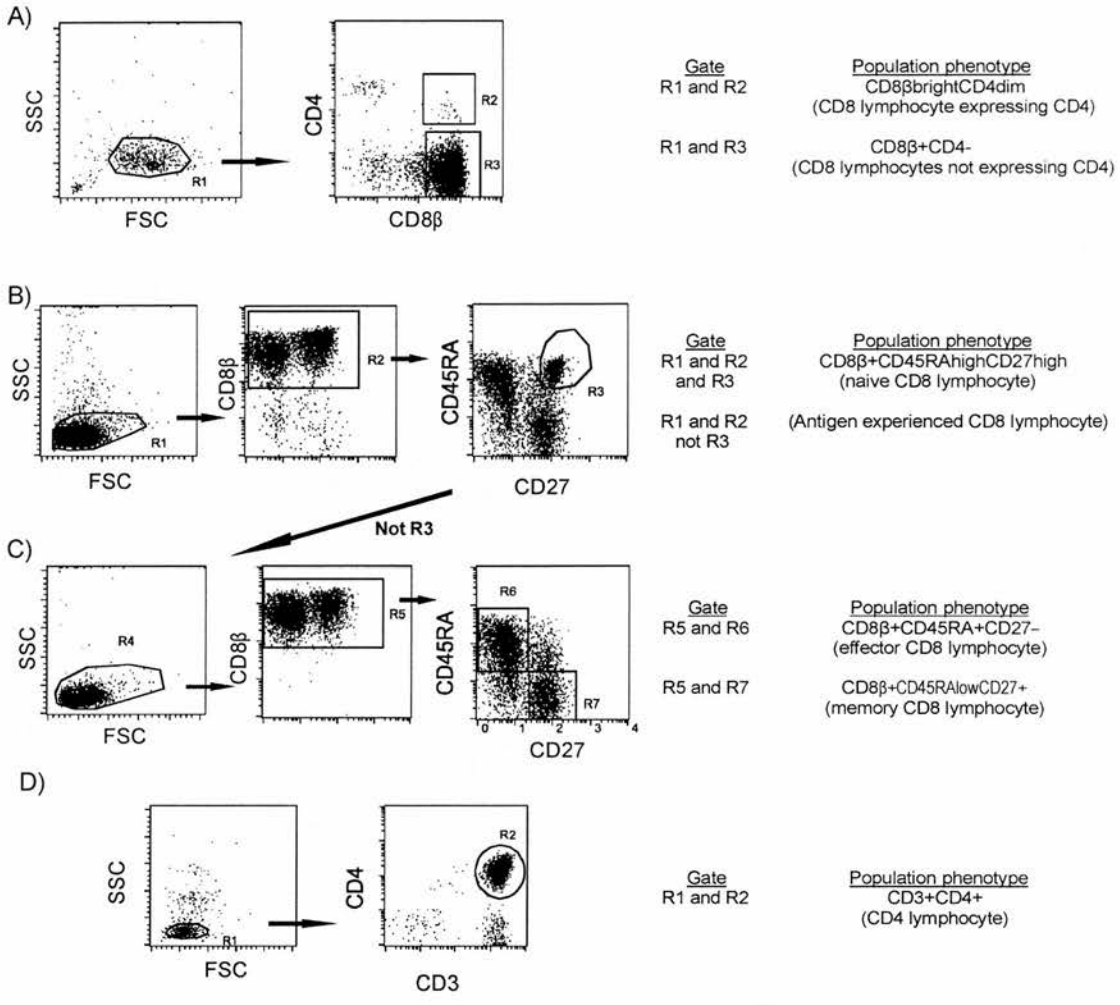


Figure 2-2. Flow activated cell sorting of CD4 lymphocytes and CD8 lymphocyte subsets. Phenotypes and gates used for fluorescent activated flow sorting of CD4 and CD8 lymphocytes are shown: CD8 lymphocytes sorted on the basis of CD4 expression (A), CD8 lymphocytes sorted on the basis of differentiation phenotype (B and C), and CD4 lymphocytes (D). Sorting on the basis of differentiation phenotype was performed in two stages, first enriched CD8 lymphocytes were sorted into antigen naïve and antigen experienced subsets (B), a portion of the antigen experienced subset was retained for HIV DNA analysis and the remainder was further sorted into memory and effector subtypes (C). (fsc = forward scatter, ssc = side scatter).

2.2 Staining cells for fluorescent activated cell sorting and flow cytometry.

FACS was used to isolate lymphocyte populations used to determine proviral load (chapter 4), and flow cytometry was used to determine the phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes (chapters 5 and 6). Staining the relevant cell populations for these processes was performed according to the following protocol. Fluorescent conjugated monoclonal antibodies were diluted to working concentrations in PBS (table 2.3). For FACS, enriched cell populations were pelleted and resuspended in 100µl AMB per 10^7 cells, then incubated for 30 minutes at 4˚C with 10µl of the appropriate fluorescent conjugated monoclonal antibodies per 10^7 cells (Table 2-1). For flow cytometry, approximately 2 x 10^6 cells were resuspended in 50µl AMB, and incubated for 30 minutes at 4˚C with 5µl of the appropriate fluorescent conjugated monoclonal antibodies, (Table 2-1). Cell were then washed in 2mls PBS, (this wash step was repeated for any tubulin stained cells), pelleted and resuspended in 0.75% w/v paraformaldehyde (Sigma Diagnostics) in PBS, then incubated at 4˚C for an hour. They were then washed twice in PBS and stored in PBS for a maximum of four days prior to flow sorting. For each experiment PBMCs were also stained with single monoclonal antibodies for use in setting flow cytometer compensation. At all times exposure of fluorescent conjugated monoclonal antibodies to light was minimised.

Fluorescent conjugated mouse anti human monoclonal antibody	Clone	Isotype	Company	Working Dilution
CD3 PE	UCHT1	IgG1,κ	BD Biosciences	1/5
CD3 Cy-Chrome™	UCHT1	IgG1,κ	BD Biosciences	1/5
CD4 Cy-Chrome™	RPA-T4	IgG1,κ	BD Biosciences	1/5
CD4 APC	RPA-T4	IgG1,κ	BD Biosciences	1/10
CD4 Alexa Fluor® 488	289-14120	IgG1	Molecular Probes	1/5
CD8β PE	2ST8.5H7	IgG1,κ	Coulter Immunotech	2/5
CD8α FITC	RPA-T8	IgG1	Pharmingen	2/5
CD14 PE	M5E2	IgG1	BD pharmingen	1/5
CD27 FITC	M-T271	IgG1,κ	BD Biosciences	2/5
CD38 FITC	HIT2	IgG1,κ	BD pharmingen	1/5
CD45RA Cy-Chrome™	HI100	IgG2b,κ	Pharmingen	1/5
CD45RO FITC	UCHL1	IgG1	Dacko	1/5
CD69 FITC	FN50	IgG1,κ	Pharmingen International	1/5
γδ-TCR PE	B1.1	IgG1,κ	Pharmingen International	1/5
Tubulin PC7	TBIA337.3	IgG1	Beckman Coulter	1/5
Isotype FITC		IgG1	Dacko	1/2
Isotype Cy-Chrome		IgG1	BD Biosciences	1/5
Isotype APC		IgG1	BD Biosciences	1/10

Table 2-3. Fluorescent conjugated monoclonal antibodies used for FACS and flow cytometry. Cy-Chrome™ is a registered trademark of Becton, Dickinson and Company. Alexa Fluor® 488 is a registered trademark of Molecular Probes.

2.3 Flow cytometry.

Flow cytometry was used to assess purity of FACS isolated cell populations, and to assess the activation and differentiation phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes. It was performed on a FACScalibur flow cytometer (Becton Dickenson) which was equipped with a 488nm argon laser and a 635nm diode laser and could measure up to four fluorescent colors, as well as fsc and ssc. It was calibrated weekly by a trained technician using calibrite beads (Becton Dickenson). Samples were acquired at approximately 1000 events per second with a threshold of 52 on the forward scatter parameter. Data was viewed and analysed using CellQuest software (BD biosciences).

2.4 Nucleic acid extraction.

DNA was isolated from the cell subsets using phenol chloroform extraction and ethanol precipitation. A negative control sample of PBMCs from a known HIV-ve subject was included in each experiment. Cells in FACS tubes were transferred into 1.5ml microtubes, with a maximum of $1.5x10^6$ cells per tube. Cells were pelleted by centrifugation at 250g for 2 minutes, the supernatant was removed and the cells were resuspended in 500µl lysis buffer (section 2.8), mixed briefly and incubated at 37° overnight. The lysed cells were then vigorously shaken for 15 minutes with 250µl chloroform (BDH Laboratory supplies, Poole) and 250µl water saturated with phenol (Rathburn Biochemicals Ltd, Walkerburn, UK), then centrifuged at 13,000 rpm for 10 minutes at room temperature (Haraeus benchtop centrifuge). The aqueous (upper) layer was transferred into a fresh 1.5ml microtube containing 500µl chloroform, mixed vigorously for 5 minutes then centrifuged at 13,000 rpm for 5 minutes at room temperature. The aqueous (upper) layer was removed and placed in a fresh 1.5 ml microtube (Maximum Recovery Microtube, Axygen Scientific Inc, CA 94587, USA), and 3M sodium acetate (pH 5.2, Sigma) and 100% ethanol were added at a ratio of 10:1:20 aqueous layer: sodium acetate: ethanol. The DNA was left to precipitate for at least 4 hours at -20°C.

The precipitated DNA was pelleted by centrifugation at 13000rpm for 15 minutes at 4°C and the supernatant removed, the pellet was washed in 70% (v/v) ethanol in water, centrifuged again (13000rpm for 5 minutes) and all supernatant carefully removed with a fine end pipette before leaving the pellet to air dry at room temperature. The pellet was then dissolved in 30 to 100µl DNA storage buffer (section 2.8) by incubating at 60°C for two hours and mixing by pipetting up and down, and stored at 4°C for immediate or next day use, or frozen at -20°C for longer term storage.

2.5 HIV provirus quantification.

HIV proviral LTR copies were quantified per µg DNA using real-time PCR and the quantification was then confirmed at limiting dilution.

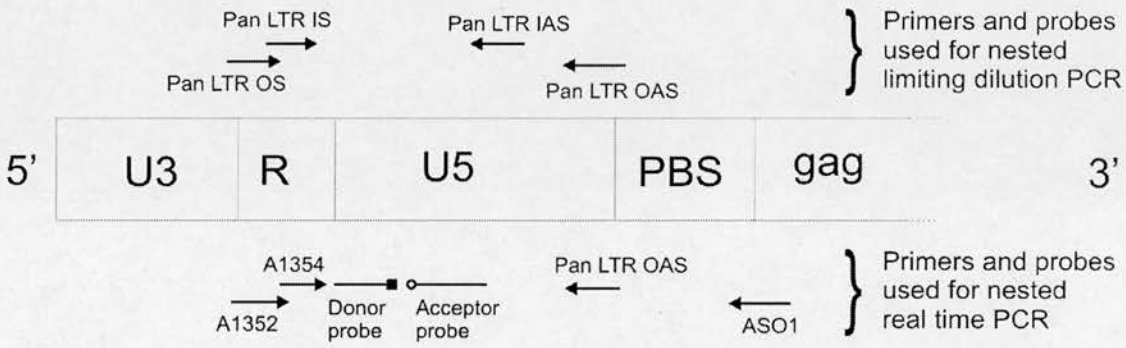


Figure 2-3 Position of primers and probes used for the real-time and limiting dilution nested PCRs shown against the HIV LTR. PBS = primer binding site.

2.5.1 Spectrophotometry

DNA was diluted 1/5 with DNA storage buffer then absorbance at 260nm wavelength was measured (corrected for absorption at 320λ) using a GeneQuant II spectrophotometer, (Amersham Biosciences, Cambridge, UK). DNA concentration was calculated using the formula: $C = A_{260} / 0.02$, where C = concentration in µg/ml,

and A_{260} = absorbance at 260nm wavelength. The A_{260}/A_{280} ratio was also checked to assess DNA purity.

Some samples contained insufficient DNA to allow spectrophotometry. In these cases the DNA concentration was estimated using the following equation:
$c = ny$; where c = concentration in μg/ml, n = the number of cells counted into the tube during FACS, and y = the mean fractional yield (extracted DNA / expected DNA) for the other samples within the same extraction run.

2.5.2 Real-time PCR.

The real-time PCR was performed using a nested approach with a conventional primary reaction and a real-time secondary and was performed with external standards. Each run included two reactions for each DNA sample, one undiluted (containing up to 150μg/ml DNA) and one diluted 1/3 with nuclease free water. Four external standards containing 5, 50, 500 and 5000 template copies, a negative control containing DNA extracted from known HIV negative PBMCs, and a negative control containing water instead of DNA were also included. The standards were DNA extracted from an HIV infected cell line diluted in DNA extracted from HIV negative PBMCs, standardized against HIV DNA of known concentration (provided by the EU Programme EVA/MRC Centralised facility for AIDS Reagents, NIBSC, Hertfordshire, UK, Grant number QLK2-CT-1999-00609 and GP828102, donated by J Bootman).

The primary reaction employed primers 'A1352': GRAACCCACTGCTTAAsscTCAA (sense) and 'ASO1': AAGCCGAGYCCTGCGTCGAGAG (antisense) (5' base positions 506 and 686 of the HXB2 genome, (

Figure 2-3). The reaction was carried out in a 20μl volume mixture containing 2μl of 10xPCR buffer (50mMKCl, 10mM Tris-HCl pH 9.0, Triton X-100 and 1.5mM $MgCl_2$), 5μM each of dGTP, dATP, dTTP and dCTP, 0.1μl sense and antisense primers (both at 10 OD), 0.4 units of *Taq* DNA polymerase (Promega Corporation, 2800 Woods Hollow Road, Madison, USA),18μl with filtered water (Milli-Q filter,

Millipore Coorporation, Bedford , USA) and 2μl DNA. The reaction was carried out on a Techne thermocycler (Techne LTD, Duxford, Cambridge, UK), and ran for 18 cycles using the following thermocycling parameters: 94°C for 18 s, 55°C for 21 s, and 72°C for 1 min 30 s, followed by a final extension step of 72°C for 6 min.

The secondary reaction used primers 'A1354': CTCAATAAAGCTTGCCTTGAG (sense) and 'pan LTR OAS': TGTTCGGGCGCCACTGCTAGAGA (antisense) (5' base positions 524 and 626 of the HXB2 genome, obtained from Oswell, Southampton, UK) and hybridisation probes LCRed705-ACTCTGGTARCTAGAGATCCCTCAGA-phosphate (acceptor probe) and AAGTAGTGTGTGCCCGTCTGTTGT-fluorescin (donor probe) (Tib Molbiol, Berlin, Germany;

Figure 2-3). The reaction was carried out in 20μ containing 2.4μl of $MgCl_2$ 25mM (Roche) (final Mg2+ concentration of 4mM), 2μl of Light Cycler DNA Master Hybridisation Probes (Roche, Mannheim, Germany), 0.4μM acceptor probe, 0.2μM donor probe, approximately 0.5μl sense and antisense primers at optical density of 10, 11.6μl PCR grade water (Roche) and 2μl of primary PCR product. The thermocycling was performed on a Light Cycler (Roche) using the following parameters: an initial 6 minute denaturation at 95°C, followed by 40 cycles of 95°C for 10 seconds, 55°C for 10 seconds and 72°C for 10 seconds, acquiring at the annealing stage.

For each reaction Roche Light Cycler software was used to calculate the crossing point using the Second Derivative Maximum method, a standard curve was drawn and the HIV LTR copies in each reaction calculated. The mean LTR copies per μg DNA was then calculated for each cell population from results obtained for the undiluted and diluted reactions.

This method was sensitive to a single copy as assessed against NIBSC HIV-1 DNA standards (provided by the EU Programme EVA/MRC Centralised facility for AIDS Reagents, NIBSC, UK, Grant number QLK2-CT-1999-00609 and GP828102, donated by J Bootman) and a single reaction was sensitive to infection frequencies of 40 proviral copies per million cells (see section 3.3.2.2).

2.5.3 *Limiting dilution PCR.*

Limiting dilution PCR was then performed to confirm the estimated proviral load using published Pan-LTR primers and thermocycling conditions (Imlach *et al.*, 2001). Pan-LTR primers were 5'-GRAACCCACTGCTTAAsscTCAA-3' (outer, sense), 5'-TGTTCGGGCGCCACTGCTAGAGA-3' (outer, antisense), 5'-CTCAATAAAGCTTGCCTTGAG-3' (inner, sense), and 5'-GAGGGATCTCTAGNYAVVAGAGT-3' (inner, antisense) (5' base positions 506, 626, 524, and 578 in the HXB2 genome, respectively; fig 2.2). Both primary and secondary reactions were carried out on a Techne Genius thermocycler (Techne LTD), using the following parameters: 94°C for 18 s, 55°C for 21 s, and 72°C for 1 min 30 s for 30 cycles followed by a final extension step of 72°C for 6 min.

Ten replicate reactions were performed at the DNA concentration expected to contain 0.5 copies per reaction. Further dilutions were performed if necessary to produce replicates containing both positive and negative results. Ten replicate reactions using the negative control DNA were included in each run. One reaction for each sample was spiked with 5 template copies to ensure that negative results were not due to the presence of inhibitors.

PCR amplicons were loaded onto a gel (2% wt/vol agarose (Seakem® LE agarose, Bioproducts, Maine, USA) in TAE buffer) containing 0.5 µg of ethidium bromide/ml. The gel was run at 150 V for 30 min and DNA visualized under UV light.

The number of LTR copies per µg DNA was then estimated from The Poisson Distribution using the program QUALITY (Rodrigo *et al.*, 1997). This assumed that the two LTRs of each provirus would segregate sperately which was only likely to occur where proviral DNA was sheared, and therefore provided a conservative estimate of LTR concentration. Standard errors were estimated using the same software according to the equation:

$SE(c) = \sqrt{\frac{2}{f''(c)}}$ where c = LTR copy number, and $f''(c)$ is 2nd derivative of chi^2 at c.

For samples where only a single positive replicate was generated due to limited availability of proviral DNA, no standard error is provided. Viral load per million cells was then estimated assuming two copies of LTR per provirus, and 6.6 µg DNA per million cells.

2.5.4 Calculation of attributable proviral load.

For each CD8 lymphocyte subset, the HIV DNA load attributable to contamination was calculated from the cell population purity data and the CD4 lymphocyte DNA load. Both 'best estimate' and 'worst case scenario' purity data are used, see section 4.3.1.2. The HIV DNA load attributable to contamination was then subtracted from the observed HIV DNA load in the CD8 lymphocyte subset to give the HIV DNA load attributable to CD8 lymphocytes. All HIV DNA loads given in the results section for CD8 lymphocyte subsets are attributable to the CD8 lymphocytes.

2.6 Laboratory layout.

The project involved determining HIV proviral load in cell populations by PCR. In order to ensure that samples did not become contaminated with PCR product, the experiments were performed in three separate laboratories, one for cell preparation, a second for extraction of DNA and primary PCR setup, and a third for secondary PCR setup.

2.7 Solutions and Buffers.

Automacs running buffer (AMB): 10% (w/v) bovine serum albumin (Sigma) plus 2mM EDTA in PBS without calcium or magnesium.

Automacs wash buffer : 2mM EDTA in PBS without calcium or magnesium.

Lysis buffer: 0.1M NaCl, 5mM tris pH 8, 1mM EDTA, 0.5% SDS, recombinant proteinase K, PCR grade 2mg/ml (Roche Diagnostics Ltd., Lewes, United Kingdom).

DNA storage buffer 10mM Tris HCl, 0.5mM EDTA (AE buffer from QIAamp DNA mini Kit, QIAGEN Ltd, Crawley, UK).

TAE: 40mM tris base (BDH laboratory supplies, Poole, England), 50mM EDTA, pH 8 (Fisher Scientific UK Ltd, Loughborough), 19mM glacial acetic acid (Sigma).

Complete medium: RPMI 1640 (Gibco, Paisley, UK), 2mmol/L glutanamine (Gibco), 100μg/ml penicillin (Gibco), and 100 IU/ml streptomycin (Gibco).

Chapter 3: Development of Methods

This project required the isolation of highly purified cell populations from limited blood samples, the extraction of nucleic acid from fixed cells and quantification of HIV proviral DNA from cell populations with infection frequencies as low as 10 copies per million cells. These requirements led to development of new cell isolation protocols, refinement of parameters for cell fixation, optimisation of phenol chloroform DNA extraction for nucleic acid recovery from fixed cells, and development of a real-time PCR for quantification of HIV provirus. For each method the rationale behind the protocol chosen is presented, and the experiments used to optimise and standardise the methodology are described.

3.1 Isolation of lymphocyte subsets

One of the major aims of this thesis was to determine the route of HIV infection of CD8 lymphocytes *in vivo*. The two most likely routes were thought to be intrathymic infection of CD8 lymphocyte precursors, or infection following CD4 upregulation during activation of mature CD8 lymphocytes. The strategy used to differentiate between these two possibilities was to determine the relative levels of HIV infection of CD8 lymphocyte subsets. Two approaches were developed. In the first, CD8 lymphocytes were divided into antigen naïve and antigen experienced subsets, with the expectation that intrathymic infection would generate HIV infected naïve cells, some of which might mature to antigen experienced status, while infection on activation would generate only HIV infected antigen experienced cells. The second approach divided cells into $CD8^{bright}CD4^{dim}$ lymphocytes and $CD8^{bright}CD4^{-}$ lymphocytes, with the expectation that infection on activation would lead to preferential infection of the former population.

To counter the possibility that HIV provirus found in CD8 lymphocyte populations could originate in contaminating CD4 lymphocytes, it was important that all CD8 lymphocyte populations were highly purified. The target level of CD4 lymphocyte contamination was less than 0.2%. Assuming a CD4 lymphocyte proviral load of

1000 copies / million cells, this level of contamination would generate two proviral copies per million CD8 lymphocytes isolated.

To allow detection of low frequency infection, the average yield of the CD8 lymphocyte subsets from a 50ml blood sample needed to be at least $1.5x10^6$ cells and cells needed to be in a condition to allow optimal DNA extraction.

The techniques available with the potential to isolate the populations of interest with sufficient yield and purity were immunomagnetic cell sorting or fluorescent activated cell sorting (FACS). Both methods require that the populations of interest be defined in terms of the expression of cell markers. In immunomagnetic sorting these markers are used to label the cells of interest with a ferrous bead, the cells are then passed through a column that is placed in a magnetic field such that the labeled cells are retained within the column and the unlabelled cells pass through. The magnetic field can then be removed and the labeled cells eluted. Sequential rounds of negative selection (where the population of interest is unlabelled) and positive selection (where the population of interest is labeled) can be used to allow selection of a cell subset defined by multiple parameters. Beads are also available that can be enzymatically removed from the cell after sorting allowing sequential positive selection, but purity is reduced for second or subsequent isolations. The number of selection steps is also limited by unavoidable loss of cells at each step, by the deterioration in cell quality with handling and by the time available. Very high purity can be achieved by repeated selection for the same label, but the gain in purity is balanced by loss of yield. Ferrous beads for immunomagnetic sorting are marketed directly conjugated to monoclonal antibodies, or two step staining methods can be used. An automated system for placing columns in and out of the magnetic field, and washing through the cell populations was available (autoMACS, Miltenyi biotec), which could be used within a negative pressure hood allowing isolation of infectious material.

For fluorescent activated cell sorting the phenotypic markers used to define the population of interest are labelled with fluorescent dyes and the labeled cells are processed through the flow sorter. The flow sorter generates a fine jet of droplets, with each droplet containing no more than a single cell. The fluorescent labels

attached to any single cell are then detected through a system of lasers and detectors which are commonly able to process three or four colours and can be set up to detect many more. In addition to fluorescent markers, cell size and shape are assessed in terms of light scatter. Forward scatter increases with increasing cell size, and side scatter with increasing irregularity of the cell shape or surface. The fluorescence and scatter parameters of each cell are then displayed digitally, and the operator can select the specific characteristics of the population to be isolated. Thus populations can be defined by degree of staining and by shape characteristics of live cells allowing much more refined population definition than is possible using immunomagnetic technique. Drops containing a cell with the specified characteristics are then electrically charged and deflected into a collection tube. The flow sorter available was not biocontained and therefore cells had to be fixed prior to sorting.

3.1.1 Isolation of antigen naïve and antigen experienced CD8 lymphocytes.

The use of phenotypic markers to define differentiation stages of CD8 lymphocytes has been a topic of much debate (Hamann *et al.*, 1999b), but the combination of CD45RA or CD45RO and either CD27, CD28, CD62L or CD11a has been shown to define a population of CD8 lymphocytes of which 85% are naïve as defined by a further three phenotypic markers (De Rosa *et al.*, 2001). CD27 and CD45RA have also been demonstrated to define populations with distinct cytokine profiles and a differentiation pathway from $CD27^{+}CD45RA^{+}$ (antigen naïve) to $CD27^{+}CD45RA^{-}$ (memory) then $CD27^{-}CD45RA^{+}$ (effector) has been proposed (Hamann *et al.*, 1997; Kern *et al.*, 1999; Hamann *et al.*, 1999a). It was therefore decided to aim to isolate naïve CD8 lymphocytes defined by CD45 isotype plus a second naïve marker.

In animal models, antigen experienced CD8 lymphocytes can revert to a naïve phenotype (Bell & Sparshott, 1990). While there is no direct evidence of this occuring in humans, there is a theoretical possibility that HIV infected phenotypically naïve CD8 lymphocytes could have acquired infection on activation rather than within the thymus. More stringent evidence for export of intrathymically infected cells would be demonstration of HIV provirus in cells recently exported

from the thymus. It has been proposed that these cells, known as recent thymic emigrants (RTE) can be identified by the expression of CD103 in addition to other naïve cell markers (McFarland *et al.*, 2000). The possibility of isolating RTE was therefore also considered.

3.1.1.1 **Immunomagnetic method.**

As cell fixing compromises DNA extraction, an immunomagnetic method was initially preferred, and a five step cell sorting strategy was drawn up which could be adapted to allow sorting of either naïve or RTE CD8 lymphocytes (Figure 3-1). The T cell isolation step was required to remove NK cells which can express CD8 and would otherwise be selected into the CD8 lymphocyte population. The MACS T cell isolation kit (Miltenyi Biotech) isolates γδ- as well as αβ-T lymphocytes. γδ-T lymphocytes have been shown to carry relatively high levels of HIV provirus ranging from 10 – 500 copies per million cells and would therefore represent a contamination issue if they were present in the CD8 lymphocyte population (Imlach *et al.*, 2003). To determine the likely levels of contamination of MACS sorted CD8 lymphocyte populations with γδ-T lymphocytes in HIV infected subjects, the proportion of γδ-T lymphocytes expressing CD8 was assessed. PBMCs were isolated from three HIV infected subjects, stained with the following fluorescent conjugated monoclonal antibodies: γδ-TCR PE, CD8α-FITC and CD3-CyChrome (table 2.3), and assessed by flow cytometry (section 2.3). γδ-T lymphocytes expressing CD8 were found to account for 0.37%, 0.89% and 6.6% of the total CD8+ve lymphocytes in the three subjects. Assuming an HIV proviral load within γδ-T lymphocytes of 10 -500 copies / million cells (Imlach *et al.*, 2003), γδ-T lymphocytes could thus generate up to 33 HIV copies / million CD8+ve lymphocytes. This level was considered unacceptable and the isolation strategy was revised to include a γδ-T lymphocyte isolation step.

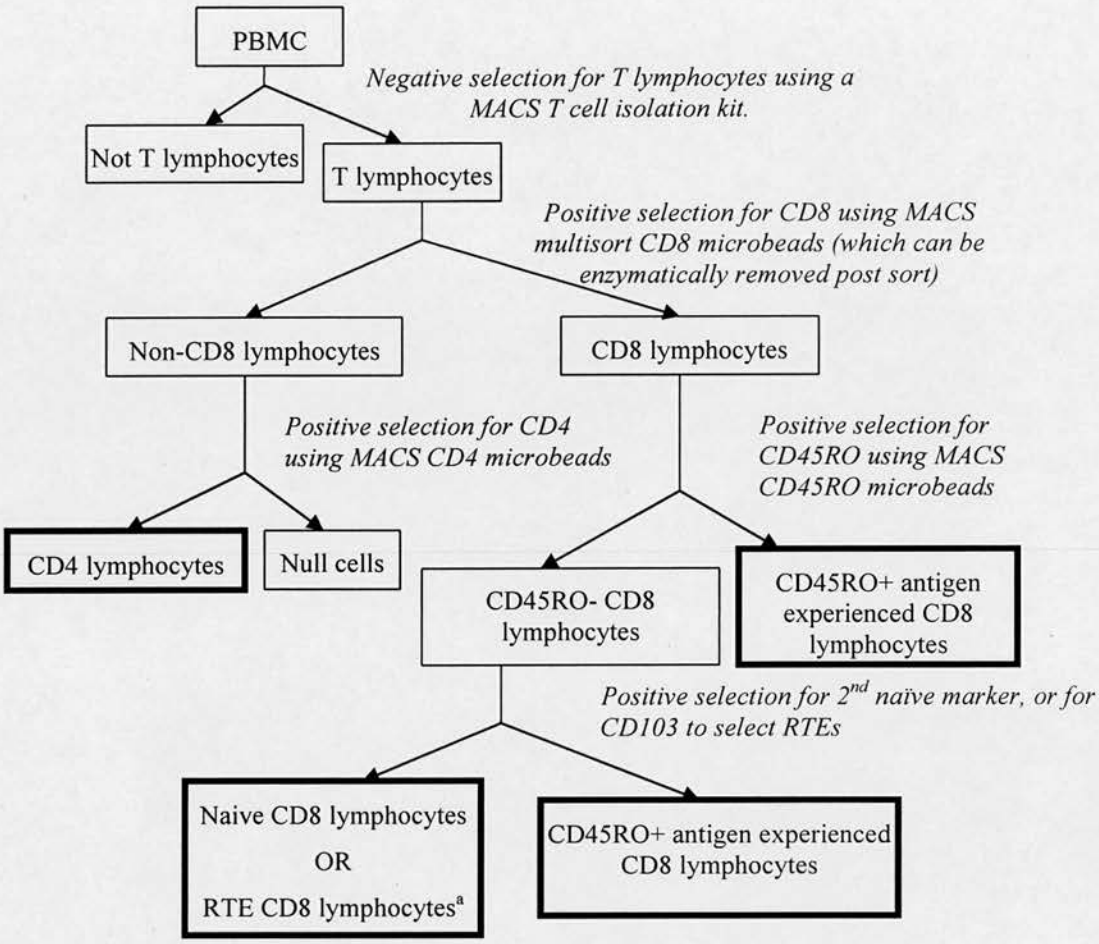


Figure 3-1. Schematic representation of the sequential immunomagnetic selection steps required to isolate naïve and antigen experienced CD8 lymphocytes and CD4 lymphocytes from blood. Target populations are shown in bold boxes. a) Recent thymic emigrant CD8 lymphocytes should be defined as CD8+CD45RO+CD62L+CD103+, this protocol would not select for CD62L thus this population would be enriched in RTE rather than a pure RTE population. MACS reagents are produced by Miltenyi Biotec.

In view of the possibility of reversion of antigen experienced cells to a naïve phenotype, the feasibility of isolating RTEs was then assessed. Using the strategy for RTE isolation given (Figure 3-1), cell subsets were isolated from a single subject. In the final selection step, cells were labeled with a FITC conjugated anti-CD103 monoclonal antibody, and then indirectly labeled with anti-FITC MACS beads, (Miltenyi Biotec). Although a relatively pure naïve CD8 population was isolated following negative selection for CD45RO (as measured by CD45RO and CD62L expression), the selection for CD103 was poor resulting in a cell population where

only 13% were CD103+ve. In addition the prevalence of CD8+CD45RO-CD62L+CD103+ cells was determined in one HIV+ve subject by flow cytometry, and found to be very low at 0.02% of all lymphocytes. It was therefore decided that isolation of these cells from HIV infected subjects was unlikely to be successful, and isolation of naive CD8 lymphocytes was prefered.

The first naïve lymphocyte marker to be assessed in addition to CD45RO was CD27, chosen as directly conjugated anti-CD27 MACs beads (Miltenyi Biotec) were available. CD27 is a recognised marker of naïve cells, but only cells expressing high levels of CD27 are true naïve, with those expressing intermediate levels having characteristics of antigen experienced cells (Kern *et al.*, 1999). Using PBMCs from three HIV infected subjects it was found that between 12% and 32% of CD8+CD45RO-CD27+ cells were CD27 intermediate, and it was thus decided that CD27 would not be a suitable 2nd marker for immunomagnetic selection of naïve cells.

Before investigating alternative naïve markers, the feasibility of the 5 step process in terms of yield and purity were assessed. The individual steps up to step 4 were optimised, and the method was tested using PBMCs from a single 50ml blood sample. Purity of the CD45RO-ve CD8 lymphocyte population was assessed by flow cytometry, and did not quite achieve the 0.2% CD4 lymphocyte contamination standard desired (Table 3-1).

Cell type (phenotype in parenthesis)	**Percentage of isolated CD8+CD45RO- population.**
CD8 lymphocytes (Lymphocyte fsc and ssc, CD8β+)	96%
CD45RO-ve cells (CD45RO-)	97%
γδ-T lymphocytes (γδ-TCR+)	0.4%
CD4 lymphocytes (Lymphocyte fsc and ssc , CD4+, CD3+)	0.5%

Table 3-1. Purity of immunomagnetically isolated CD8+CD45RO- population. Fsc = forward scatter, ssc = side scatter.

Typical cell losses during the procedure were 20% of cells at each +ve selection step and 30% at each –ve selection step, giving projected cumulative losses over a five step process of 78%. The number of naïve CD8 lymphocytes present in four HIV +ve subjects was assessed and ranged from $3.9x10^4 – 1.4x10^5$ cells per ml of blood. With 78% losses this would lead to isolation of between $4.3x10^5$ and $1.5x10^6$ naïve CD8 lymphocytes per 50ml sample, barely sufficient for both purity checks and proviral load estimation.

3.1.1.2 **Two stage method of immunomagnetc enrichment followed by FACS.**

Given the problems outlined above, the alternative strategy of FACS was investigated, and the concerns regarding proviral quantification from fixed tissue were approached by optimising the fixation and DNA extraction methods (section 3.2). A FACSVantage flow sorter was available (Becton Dickenson) equipped to sort two retained populations and a waste population based on forward scatter, side scatter, and three fluorescent markers.

The initial strategy, tested using blood donated by an HIV infected subject, was to isolate PBMCs from blood by density centrifugation, then split the PBMCs into two aliquots, one for isolation of antigen naïve and antigen experienced CD8 lymphocytes and the other for CD4 lymphocyte isolation. The phenotypes and gates used to define these populations are the same as those used in the final protocol (2.1.4). The two PBMC aliquots were stained with appropriate fluorescent conjugated monoclonal antibodies (Table 3-2).

Population to be isolated	Monoclonal antibodies
Antigen naïve and antigen experienced CD8 lymphocytes	CD27 FITC CD8β chain PE CD45RA Cy-chrome™ CD4 APC
CD4 lymphocytes	CD3 PE CD4 Cy-chrome™

Table 3-2. Fluorescent conjugated monoclonal antibodies used to stain PBMC for FACS. See table 2.3 for clones and manufacturers of the antibodies.

The purity of the naïve and antigen experienced CD8 lymphocytes was assessed on a facscalibur flow cytometer that was able to detect the fluorochrome APC (allowing assessment of contamination with CD4 lymphocytes) in addition to the three fluorochromes used for sorting (section 2.3). The yields of naïve and experienced CD8 lymphocytes were adequate at $7.7x10^5$ and $2x10^6$ respectively, but the CD4 contamination level of the naïve subset was too high at 0.41%.

Contamination of cell populations isolated by FACS with cells from outside the selection gate is usually due to the presence of the unwanted cell in the droplet immediately following a desired cell. In this situation the unwanted droplet is often deflected along with the correct droplet. The easiest way to avoid this is to sort the cells in a more dilute suspension such that the possibility of two consecutive droplets containing cells is diminished, but this can lead to very long sorting times with cost implications. An alternative approach is to pre-enrich for the desired population, and this approach was taken by introduction of an immunomagnetic CD8 lymphocyte enrichment step prior to the FACS. This also had the beneficial effect of increasing the yield of CD8 and CD4 lymphocytes. The strategy proved successful in fulfilling all five of the initial requirements and no further refinements were made. The method is given in full in section 2.1 and yield and purity data are presented in section 4.3.1.

3.1.2 Isolation of $CD8^{bright}CD4^{dim}$ lymphocytes and $CD8^{bright}CD4^{-}$ lymphocytes.

3.1.2.1 **Immunomagnetic method.**

$CD8^{bright}CD4^{dim}$ lymphocytes and $CD8^{bright}CD4^{-}$ lymphocytes could be isolated by immunomagnetic selection through use of a negative selection for T cells, followed by positive selection for CD8 and then positive selection for CD4 (Figure 3-2). This strategy has a number of problems. Firstly, it would result in selection of all CD8+CD4+ lymphocytes, including $CD4^{bright}CD8^{dim}$ (thought to originate from CD4 lymphocytes) as well as $CD8^{bright}CD4^{dim}$, and second all CD4 lymphocytes contaminating the CD8 lymphocyte population would be selected into the CD8+CD4+ population.

To assess whether $CD4^{bright}CD8^{dim}$ lymphocytes were likely to result in significant contamination of the $CD8^{bright}CD4^{dim}$ population, the relative numbers of $CD4^{bright}CD8^{dim}$ and $CD8^{bright}CD4^{dim}$ lymphocytes was checked in three healthy subjects. One subject demonstrated a substantial population of $CD4^{bright}CD8^{dim}$ lymphocytes, sufficient to cause a major purity issue for immunomagnetic isolation (Figure 3-3).

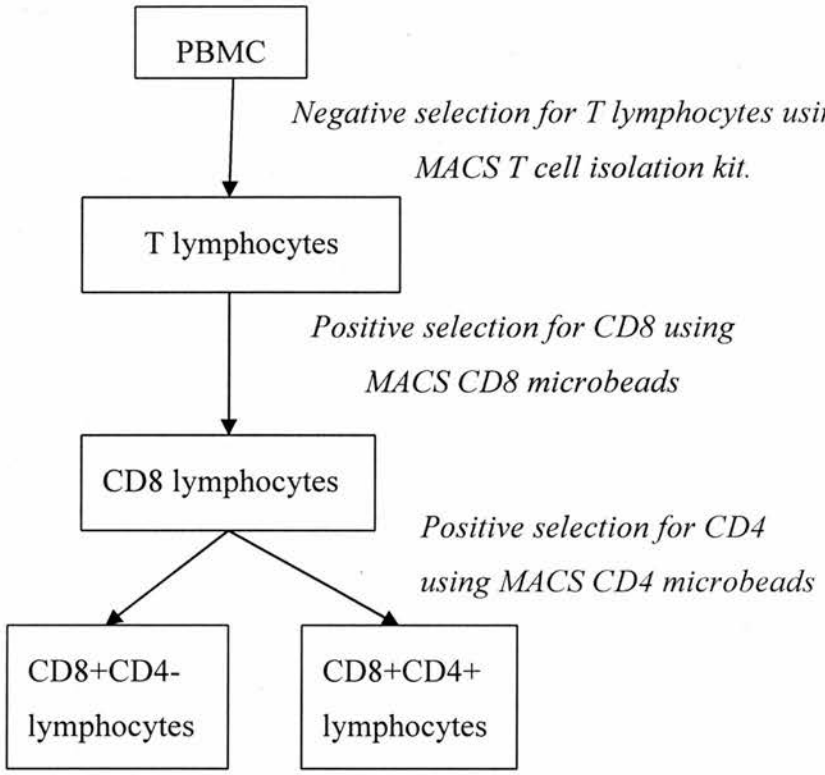
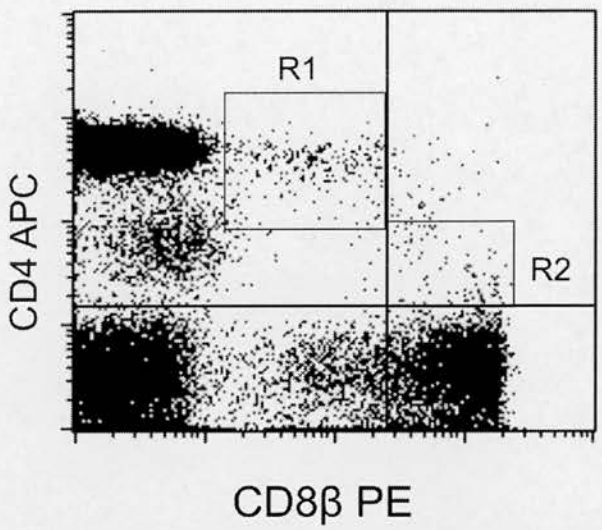


Figure 3-2. Schematic diagram of an immunomagnetic approach for isolation of $CD8^{bright}CD4^{dim}$ and$CD8^{bright}CD4^{-}$ lymphocytes. MACS reagents are produced by Miltenyi Biotec.



Region	Number of cells
R1	112
R2	41

Figure 3-3. Relative prevalence of $CD8^{bright}CD4^{dim}$ and $CD4^{bright}CD8^{dim}$ lymphocytes. Dot plot of PBMCs from a healthy subject, gated on lymphocyte forward and side scatter characteristics and stained for CD4 and CD8. In this subject there are more $CD4^{bright}CD8^{dim}$ lymphocytes (R1) than $CD8^{bright}CD4^{dim}$ lymphocytes (R2).

The likely extent of CD4 lymphocyte contamination of an immunomagnetically isolated CD8+CD4+ lymphocyte population was calculated from published results. Previous levels of CD4 contamination of CD8 lymphocyte populations isolated using MACS beads were 0.2 - 0.5% (McBreen *et al.*, 2001; Imlach *et al.*, 2001). Given that $CD8^{bright}CD4^{dim}$ lymphocytes usually represent 0.4 - 3.4% of CD8 lymphocytes in HIV infected subjects (Zloza *et al.*, 2003), co-localisation of the $CD8^{bright}CD4^{dim}$ lymphocytes and the contaminating CD4 lymphocytes would result in a population comprising 5 – 55% CD4 lymphocytes. This level of CD4 lymphocyte contamination would be unacceptable.

3.1.2.2 **Two stage method of immunomagnetc enrichment followed by FACS.**

In view of the problems inherent in immunomagnetic isolation of $CD8^{bright}CD4^{dim}$ lymphocytes outlined above, the same two stage approach described for separation of naïve and experienced CD8 lymphocytes (immunomagnetc enrichment followed by

FACS, 3.1.1.2) was adopted for isolation of $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes.

Flow cytometry is notorious for detection of small populations of double positive cells that are an artefact of the staining or detection process. (http://www.cyto.purdue.edu/hmarchiv/2001/2463.htm)(Ekong *et al.*, 1993; Nicholson *et al.*, 1994). Such populations may be caused by non-specific staining, increased autofluorescence, coincidence error or cell clumping.

To ensure the $CD8^{bright}CD4^{dim}$ events were not simply CD8 lymphocytes with increased non-specific staining, the non-specific staining properties of the APC- and Cychrome- conjugated anti-human CD4 monoclonal antibodies were mimicked using appropriate isotype controls (Table 2-3). Very little non-specific staining was seen, and a clear increase in staining was observed when the anti-CD4 monoclonal antibodies were used.

Dead cells can auto-fluoresce and bind non-specifically to monoclonal antibodies. The vast majority of dead cells were excluded from the sorted populations by gating on forward and side scatter characteristics of live lymphocytes. For four of the HIV infected subjects, the population to be sorted into $CD8\beta^{bright}CD4^{dim}$ and $CD8\beta^{bright}CD4^{-}$ lymphocytes was co-stained with an anti-tubulin monoclonal antibody to further exclude dead cells. Unlike live cells, dead cells are porous to antibody and will thus bind anti-tubulin antibody (tubulin being one of the major components of the cytoskeleton). In these subjects the frequency of tubulin stained cells within the $CD8\beta^{bright}CD4^{dim}$ lymphocyte population (defined using a tight lymphocyte fsc and ssc gate) was reassuringly low, although only small numbers of events were acquired (Table 3-3).

Coincidence error occurs when two cells present in a single droplet, lie behind one another as viewed from the fsc and ssc detectors thus mimicking a single cell. This could result in a single CD4 lymphocyte together with a single CD8 lymphocyte appearing as a CD8+CD4+ event. This type of artefact was of particular concern as

it could lead to high levels of CD4 lymphocyte contamination. The frequency of coincidence was therefore assessed using two approaches, first using a separate staining then mixing experiment, and second by sorting $CD8^{bright}CD4^{dim}$ lymphocytes and then checking their identity by flow cytometry.

Subject	Fraction of $CD8^{bright}CD4^{dim}$ lymphocytes that stain for tubulin
6	0/15
11	0/113
14	1/34
19b	0/410

Table 3-3. Dead cells within the $CD8^{bright}CD4^{dim}$ subset. The proportion of $CD8^{bright}CD4^{dim}$ lymphocytes staining for tubulin in four HIV infected subjects. The lymphocytes were tightly gated by forward and side scatter characteristics.

In the first experiment PBMCs from a healthy donor were divided into aliquots and stained either for CD8β (aliquot A) or CD4 (aliquot B) or both CD8β and CD4 (aliquot C). All cells were also stained for CD27 and CD45RA to mimic the conditions used in subsequent experiments. Three samples were prepared for flow cytometry, sample 1 contained a 1:1 mixture of aliquots A and B mixed immediately after staining and incubated at 4° overnight, sample 2 contained a 1:1 mixture of aliquots A and B mixed immediately prior to flow cytometry, and sample 3 contained aliquot C cells only. In the text to follow, samples 1 and 2 are referred to as 'mixed' while sample 3 is refered to as 'co-stained'. Appropriate isotype controls were also included. If the $CD8^{bright}CD4^{+}$ population were the result of coincidence error, it would be seen in the 'mixed' as well as the 'co-stained' sample.

The frequency of $CD8^{bright}CD4^{+}$ cells in the 'mixed' samples was found to be very low (mean 0.2% of CD8 lymphocytes, range 0.1 - 0.24), contrasting well with the clear double positive population seen in the 'co-stained' sample (Figure 3-4A). However, as the population of $CD8^{bright}CD4^{+}$ lymphocytes in the 'co-stained' sample was also small (2.7% of CD8 lymphocytes), coincidence error could still have accounted for 10% of the $CD8^{bright}CD4^{+}$ lymphocytes. Coincidence error is not the

only explanation for the appearance of $CD8^{bright}CD4^{+}$ events in the 'mixed' samples, which could be due to carry over of small amounts of unbound anti CD4 antibody from the CD4 stained sample into the mixed sample. This explanation is supported by the following: a) the $CD8^{bright}CD4^{+}$ events in the mixed sample had very low intensity CD4 expression, and no $CD8^{bright}CD4^{bright}$ events were observed (Table 3-4, Figure 3-4B); b) the number of $CD8^{bright}CD4^{+}$ events did not increase with increasing speed of acquisition (Table 3-4); c) there was no increase in $CD8^{bright}CD4^{+}$ cells in the sample mixed then incubated overnight (which could promote cell adhesion) than in the sample mixed immediately prior to flow cytometry (Table 3-4).

The extent of coincidence error was also assessed by checking the purity of $CD8^{bright}CD4^{dim}$ lymphocytes isolated by FACS from five HIV positive subjects. Any cells sorted erroneously due to coincidence error would be expected to appear as separate CD4 and CD8 lymphocytes in the purity check. The median contamination by CD4 lymphocytes was 0.3% (Table 4.3A), arguing against significant coincidence error.

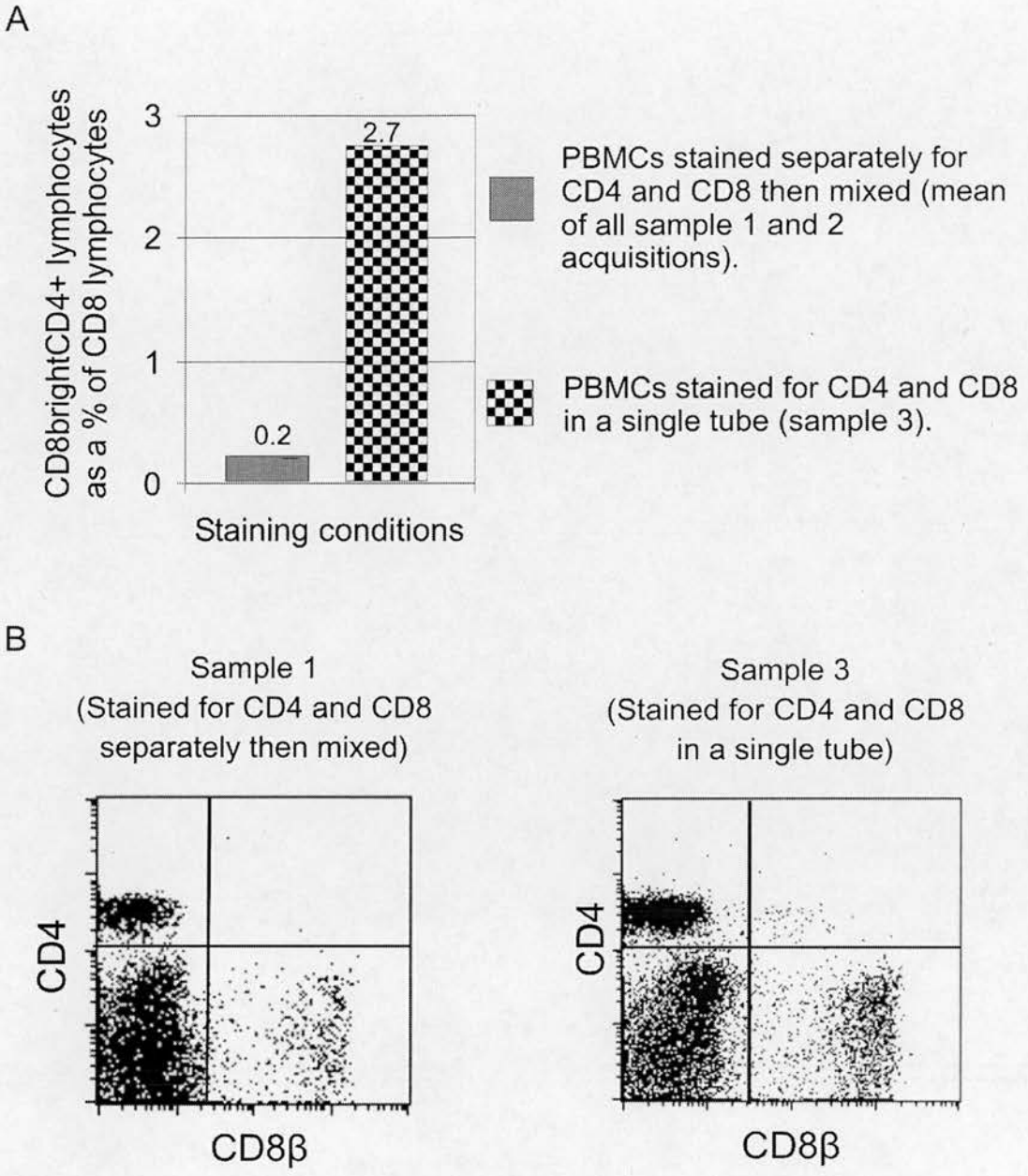


Figure 3-4. Role of coincidence error in generation of $CD8^{bright}CD4^{dim}$ events. The impact of coincidence error was assessed by determining the frequency of $CD8^{bright}CD4^{+}$ lymphocytes in samples that contained a mixture of cells separately stained for CD4 or CD8 (samples 1 and 2, 'mixed samples') compared to their frequency in samples stained for CD4 and CD8 in a single tube (sample 3, 'co-stained' sample). (A) The bar chart indicates the mean frequency of $CD8^{bright}CD4^{+}$ lymphocytes from all flow cytometry acquisitions of the mixed samples (grey bar) and the 'co-stained' sample (chequered bar). (B) Dot plot of CD4 against CD8 expression for samples 1 and 3, (gated with a tight gate on lymphocyte forward and side scatter characteristics) showing the lack of double stained cells in the 'mixed' compared to the 'co-stained' sample. (This subject consistently displayed a significant $CD4^{bright}CD8^{dim}$ population).

Cell clumping occurs when the cell preparation does not form a single cell suspension, but one or more cells stick together. A doublet of a CD4 lymphocyte and a CD8 lymphocyte could present a CD8+CD4+ appearance on flow cytometry, and if cells were well adhered this appearance could be maintained after sorting. Such doublets are generally excluded by gating on the fsc/ssc properties of single cells. To ensure that the $CD8^{bright}CD4^{dim}$ population did not represent doublets mimicking single cells, an aliquot of sorted $CD8^{bright}CD4^{dim}$ cells were viewed under light microscopy. Of 100 cells viewed, all had the appearance of single cells.

Sample	Speed of acquisition (events / second)	$CD8^{bright}CD4^{+}$ as % of CD8 lymphocytes	$CD8^{bright}CD4^{dim}$ as % of CD8 lymphocytes	$CD8^{bright}CD4^{bright}$ as % of CD8 lymphocytes
Sample 1	400	0.23	0.18	0.05
Sample 2	300	0.24	0.27	-0.03
	450	0.10	0.03	0.07
	675	0.24	0.20	0.04
Mean of sample 1 and sample 2 data		0.20	0.17	0.03
Sample 3	450	2.73	1.11	1.62

Table 3-4. Coincidence error. The likelihood that coincidence error was the cause of the small number of $CD8^{bright}CD4^{+}$ lymphocytes seen in 'mixed' samples was assessed by altering speed of acquisition, and by quantifying the number of $CD8^{bright}CD4^{bright}$ as compared to $CD8^{bright}CD4^{dim}$ events. Coincidence error would be expected to increase with increasing speed of acquisition, and to generate $CD8^{bright}CD4^{bright}$ in preference to $CD8^{bright}CD4^{dim}$ events. These trends were not observed suggesting alternative explanations for the $CD8^{bright}CD4^{+}$ lymphocytes seen in 'mixed' samples.

In view of the data indicating that the $CD8^{bright}CD4^{dim}$ events apparent on flow cytometry were not artefactual, a method of immunomagnetic enrichment followed by FACS was used to isolate $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes from test subjects. The full protocol is given in section 2.1.

3.2 Cell fixation and DNA extraction.

Due to aerosol generation and infection risk during FACS, cells had to be fixed prior to sorting. All methods of fixation have some effect on DNA, and as HIV proviral load was to be assessed in the sorted cells, it was important to develop a fixation method that allowed maximal DNA extraction and accurate proviral load quantification.

The standard fixation protocol used prior to cell sorting was to incubate cells for one hour in 2% paraformaldehyde. Paraformaldehyde damages DNA by initiating denaturation at AT rich regions creating sites for chemical interaction (Douglas & Rogers, 1998). It can also hamper DNA extraction by cross-linking histone proteins. The damage to DNA can be reduced by altering a number of fixation parameters including reduction of paraformaldehyde concentration (McGhee & von Hippel, 1977), ensuring a neutral pH (Douglas & Rogers, 1998), fixing at low temperature (Tokuda *et al.*, 1990; Yagi *et al.*, 1996; Noguchi *et al.*, 1997) fixing in the presence of EDTA (Yagi *et al.*, 1996), and reducing the duration of fixation. Alternatively use of alcoholic fixatives such as 100% ethanol or methanol have good nucleic acid preservation properties (Noguchi *et al.*, 1997). The approach taken first was to optimise the paraformaldehyde fixation parameters, and if this failed then alternative fixatives were to be trialed.

3.2.1.1 **Duration of fixation.**

PBMCs from an HIV infected subject were fixed in 2% paraformaldehyde for either 10 or 20 minutes. DNA from duplicate samples of the fixed cells and unfixed controls was then extracted and HIV proviral load quantified using the LightCycler real-time PCR assay described in 2.5.2. Both 10 and 20 minutes fixation lead to a marked decreased in provirus detection (Figure 3-5).

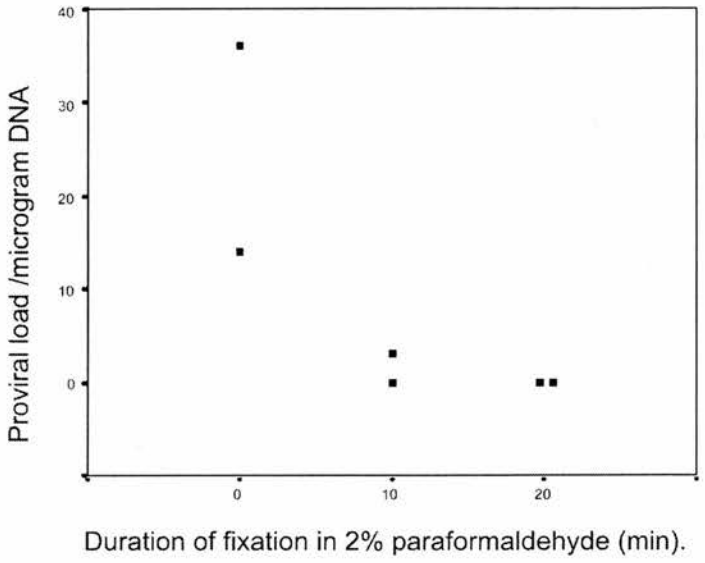


Figure 3-5. Effect of duration of fixation on proviral load detection. HIV proviral load detected in $1x10^6$ PBMCs isolated from an HIV infected subject and fixed in 2% paraformaldehyde for 0, 10 or 20 mins (duplicate samples).

3.2.1.2 **Concentration of paraformaldehyde.**

To determine whether use of a less concentrated paraformaldehyde solution would allow improved DNA recovery, DNA was extracted from HIV infected C8166 cells fixed for 1 hour in varying concentrations of paraformaldehyde. HIV provirus was then quantified from a $1x10^{-5}$ dilution of the extracted DNA using real-time PCR. (The infection of the C8166 cells and the DNA extraction was performed by Dr. Imlach). No provirus was detected in cells fixed in 2% or 1.75% praformaldehyde, while concentrations up to 0.5% had no detrimental effect (Figure 3-6).

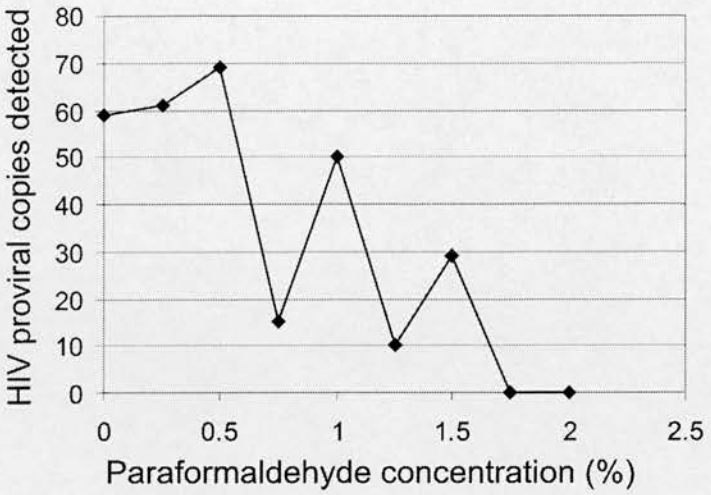


Figure 3-6. Effect of paraformaldehyde concentration on ability to detect HIV provirus. HIV infected C8166 cells were fixed in various concentrations of paraformaldehyde, DNA was extracted and HIV proviral load quantified by real-time PCR.

3.2.1.3 Phenol chloroform DNA extraction method for paraformaldehyde fixed cells.

The standard phenol chloroform based DNA extraction methods consists of a lysis step followed by DNA extraction and ethanol precipitation. It was expected that the cell fixation process would compromise the ability of the lysis step to fully separate the DNA from the protein component, and therefore the standard lysis method was compared against two enhanced methods. Stored PBMCs isolated from a HIV infected subject were thawed, washed, resuspended in 1 X PBS, filtered to ensure a single cell suspension and divided into 24 aliquots each containing $1x10^6$ cells. Six aliquots were fixed with either 0%, 0.75%, 1% or 1.25% paraformaldehyde and DNA was extracted in duplicate using three protocols: 'standard lysis', 'hot lysis' or 'extended lysis'. All methods involved lysis with SDS and proteinase K. In the hot lysis method a PBS based lysis buffer was used and cells were heated to 100°C for 10 minutes prior to a 12 hour incubation at 56°C, in the standard and extended protocols the lysis buffer was TNE based with no high temperature step and the methods differed only in the length of the lysis period (standard = 2 hours, extended = 12 hours).

It was found that separation of the aqueous and organic layers was much easier in the hot lysis than in the other two methods due to decreased viscosity of the aqueous layer. The amount of DNA extracted using each method was measured by 260nm wavelength light absorption (corrected for absorption at 320λ) using a GeneQuant II spectrophotometer. Combining all samples the extended lysis protocol was significantly superior to the other two (Figure 3-7A) and this superiority was evident for all paraformaldehyde concentrations tested (Figure 3-7B).

The ratio of the light absorbance at wavelength 260nm to that at 280nm gives a measure of the DNA purity, with a ratio of 1.8 being optimum and lower levels indicating significant protein contamination. None of the extraction methods demonstrated ratios suggestive of protein contamination even at the higher paraformaldehyde concentrations. The higher ratios seen for the standard lysis conditions may reflect increased RNA retention or phenol contamination, (Figure 3-7C).

HIV proviral load per μg DNA extracted using the 'hot' and 'extended' lysis conditions was quantified using real-time PCR (fig 3.2.3.D). Neither method showed overall superiority, though the 'hot' method did appear to give more consistent results. It is possible that this superior consistency could be due to the easier separation of aqueous and organic layers due to denaturing and shearing of DNA during the 100°C incubation.

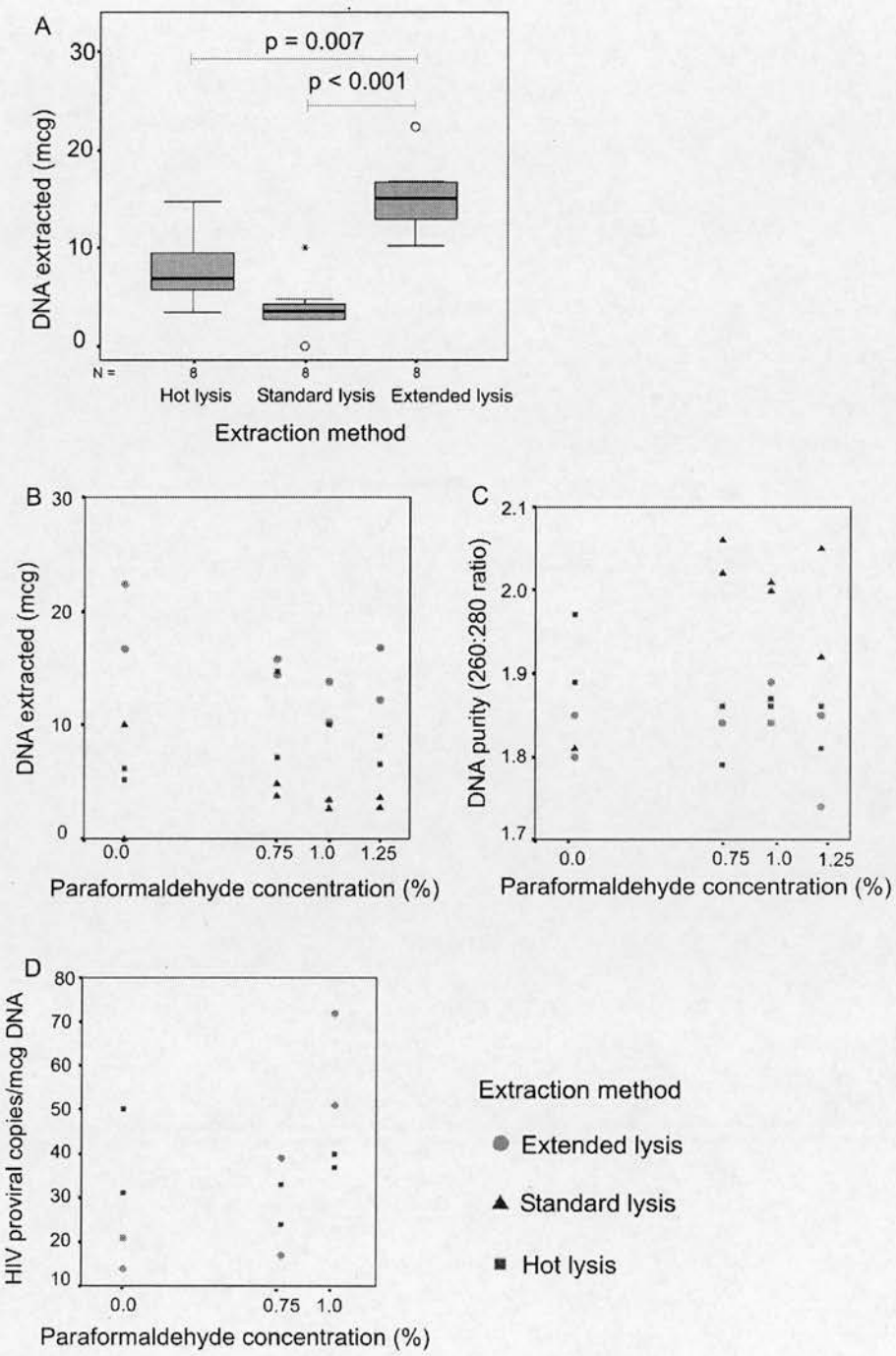


Figure 3-7. Comparison of lysis conditions for extraction of DNA from fixed cells. PBMCs were fixed in 0.75%, 1.0% or 1.5% paraformaldehyde or left unfixed, and DNA extracted using one of three different lysis conditions followed by phenol chloroform extraction and ethanol precipitation. The amount of DNA extracted was measured by spectrophotometry. Overall DNA extraction efficiency was significantly greater using extended lysis conditions (A), this was evident across all paraformaldehyde concentrations used (B); DNA purity was high as measured by the ratio of absorbance at 260λ:280 λ (C). There was no consistent difference between the hot and extended lysis conditions in the number of HIV proviral copies detected per μg DNA (D).

Finally, given that cell numbers available for some of the CD8 lymphocyte subpopulations were expected to be low, the DNA yield using the extended and hot methods was compared at low cell numbers. PBMC samples containing either $2.1x10^5$ cells or 1.4×10^6 cells (the lower cell number was included to mimic the conditions expected for CD8 lymphocyte subsets) were fixed in 0.75% paraformaldehyde for an hour then DNA was extracted using either 'hot' or 'extended' lysis conditions. Again the DNA yield was consistently higher in the 'extended' as compared to the 'hot' protocol, (Figure 3-8). Extraction failed in one sample from the 'hot' lysis arm.

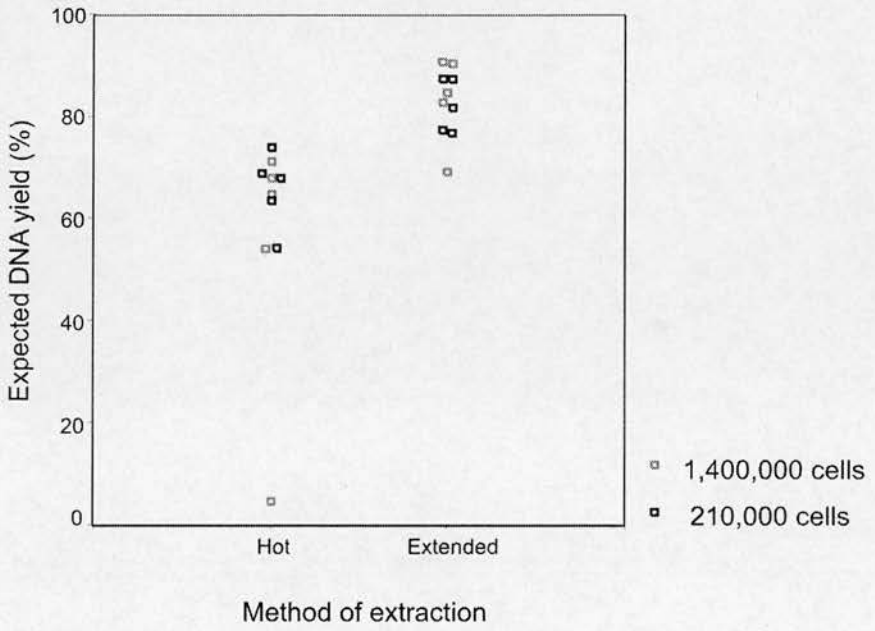


Figure 3-8. Effect of lysis conditions on DNA yield from small populations of cells. DNA yield was superior using 'extended' as compared to 'hot' lysis conditions when extracting from high or low numbers of cells.

3.2.1.4 **Infectivity of HIV infected cells fixed with 0.75% paraformaldehyde.**

To ensure that the fixation with 0.75% paraformaldehyde for an hour at 4°C was adequate to render HIV infected cells non-infectious (and safe to sort on the flow sorter), the infectivity of an HIV infected T cell line, fixed using the above parameters was tested. This work was performed by Dr. S. Imlach, (Laboratory of

Clinical and Molecular Virology, University of Edinburgh) and demonstrated that the fixation protocol was sufficient.

3.3 Quantification of HIV provirus.

Quantification of HIV proviral load in cell subsets has traditionally been performed by limiting dilution PCR (Simmonds *et al.*, 1990). In this method a nested PCR sensitive to a single HIV proviral copy is performed on serial dilutions of DNA extracted from the cell population of interest. Multiple replicates are performed at the dilution expected to contain just less than a single copy (on average) per reaction, and the true average number of copies per reaction is calculated from the number of positive and negative replicates using an equation derived from the Poisson Distribution. This method is very sensitive (its sensitivity being limited only by the amount of DNA available), but is very labour intensive. With the aim of increasing time efficiency a real-time PCR quantification method was developed. The HIV LTR was chosen as the target sequence as it is highly conserved between different HIV subtypes. This was a priority as many of the HIV infected subjects available for recruitment to the study had acquired the virus outside the UK and inclusion of subjects with non-subtype B virus in the study population was likely. The real-time PCR reaction was performed on a Roche Light Cycler (Roche) using hybridization probes and external standards. Initial primers, probes and reaction conditions were based on those developed for detection of HIV RNA in pooled plasma (Cleland *et al.*, 2001).

3.3.1 Quantification of HIV provirus using real-time PCR.

3.3.1.1 **Sensitivity.**

Using two dilution series the relative sensitivity of a single round real-time PCR was compared with a nested approach using a standard primary reaction and a real-time secondary reaction. The first dilution series used DNA extracted from an HIV infected cell line, while the second used cloned HIV proviral DNA. Both series were diluted in DNA extracted from HIV negative PBMC at a concentration of 600µg/ml (1.2µg DNA per PCR, DNA concentration of 60µg/ml in PCR reaction). DNA

extracted from PBMCs was used as the diluent instead of water or carrier DNA to approximate the conditions when testing proviral load in cell subsets isolated from HIV infected subjects. The high DNA concentration was chosen as it would allow the proviral load of approximately $5x10^5$ cells to be determined in one reaction, though as a result of experiments assessing inhibiton of PCR (3.3.1.2), the concentration of DNA in standards was subsequently reduced to 100µg/ml. Using both dilution series the nested approach was shown to be 1 log more sensitive than the single round.

To determine the actual sensitivity (in terms of number of provirus copies in a reaction required for detection) a standard series of known proviral copy concentration was generated by diluting a known amount of HIV proviral clone (measured by spectrophotometry). The LTR concentration in the standard was also assessed using limiting dilution, which demonstrated that $1x10^{-10}$ dilution of the clone contained 0.6 LTR copies per 2µl (Figure 3-9).

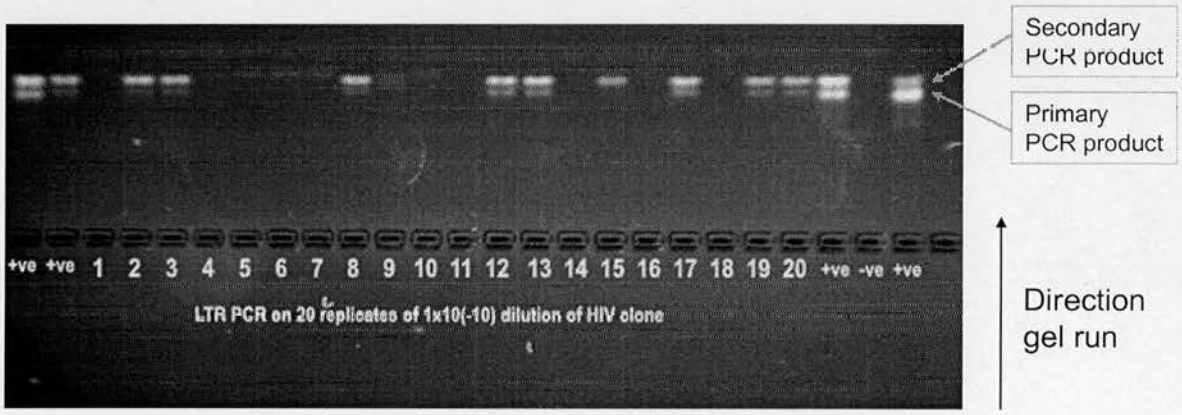


Figure 3-9. Quantification of LTR copies in standard using limiting dilution PCR. The LTR concentration in the 10^{-10} dilution of the standard (full length HIV clone diluted in DNA extracted from HIV negative PBMCs) was determined by limiting dilution. Nine out of twenty replicates were positive indicating an LTR concentration of 0.6 copies per 2µl.

The proviral concentration of the standards was also checked by limiting dilution using primers against HIV gag (Simmonds *et al.*, 1990), and results correlated well with those obtained using LTR primers. The accuracy of the gag limiting dilution

method was confirmed against a standard series of HIV proviral DNA at known concentration (provided by the EU Programme EVA/MRC Centralised facility for AIDS Reagents, NIBSC, Hertfordshire, UK, Grant number QLK2-CT-1999-00609 and GP828102, donated by J Bootman). The NIBSC standard lacked the LTR and thus could not be used directly to assess the LTR quantification methods.

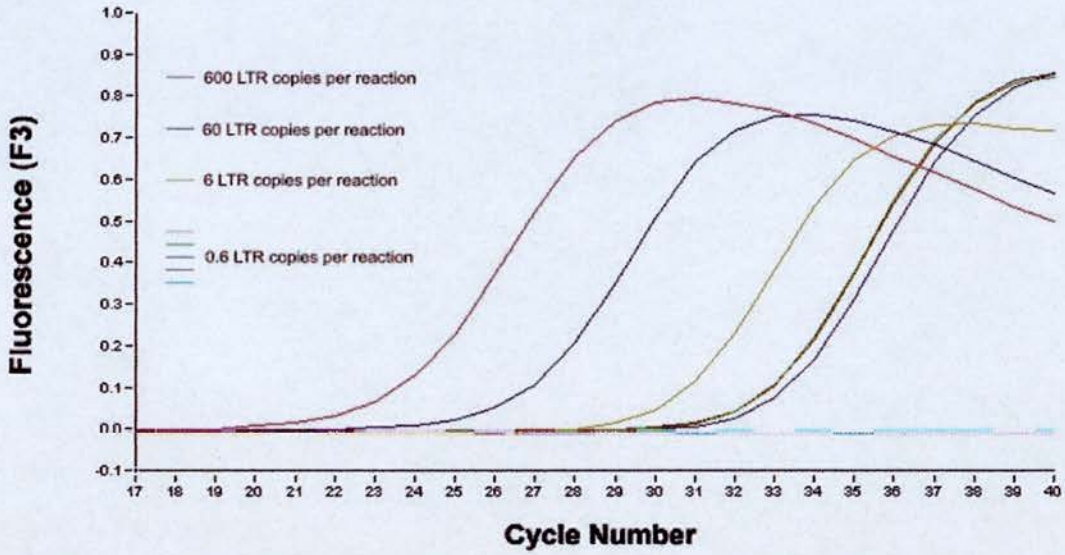


Figure 3-10. Sensitivity of real-time PCR for LTR. The sensitivity of the nested real-time PCR protocol was tested using in house standards generated by serial dilution of a full length HIV clone diluted in DNA extracted from HIV negative PBMCs at a concentration of 100µg/ml. The nested real-time PCR protocol was positive in 3/5 replicate reactions containing an average of 0.6 LTR copies each (as assessed by limiting dilution, Figure 3-9).

The sensitivity of the 18 cycle conventional primary and real-time secondary PCR was then tested and was found to be approximately five copies, which was considered insufficient for the purpose. To improve sensitivity, the probes were altered to optimise binding to M group virus, a 'hot start' real-time reaction was used, and the amount of carrier DNA in the dilution series was reduced to 100µg/ml. Following these changes the protocol was consistently sensitive to a single LTR copy (Figure 3-10). Full details of the final protocol, probes and primer sequences is given in section 2.5.2.

3.3.1.2 Determining the optimum DNA concentration for proviral load quantification.

As the expected number of proviral copies in the cell populations of interest was very low, ranging from less than 1 to 1000 copies per million cells (Livingstone *et al.*, 1996; McBreen *et al.*, 2001), the real-time PCR assay was required to assess DNA extracted from a large number of cells (up to 10^6). Too high a concentration of DNA in each PCR leads to inhibition, while use of a low concentration would necessitate multiple replicate reactions with cost implications. To determine the maximum DNA concentration tolerated without compromising the reaction efficiency, dilution series of HIV provirus were made up in DNA extracted from HIV negative PBMCs at concentrations of 150, 300 and 900 µg/ml; giving final DNA concentrations in the PCR reaction mix of 15, 30 and 90µg/ml respectively. The crossing points (the number of real-time PCR cycles required to generate a given amount of template) for the 15µg/ml DNA and 30µg/ml DNA reactions were almost identical, but those for the 90 µg/ml DNA were delayed demonstrating inhibition of the PCR reaction at this concentration (Figure 3-11).

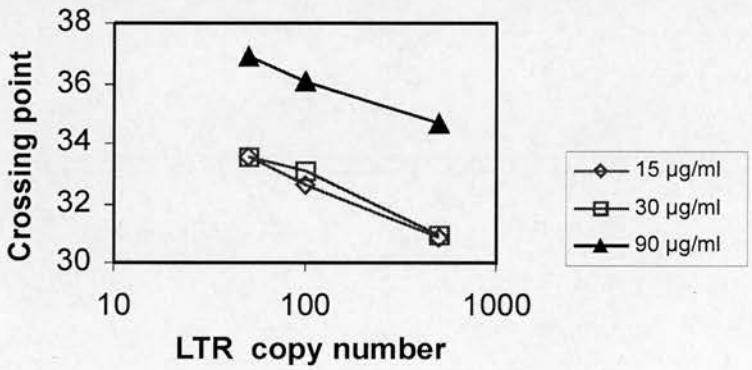
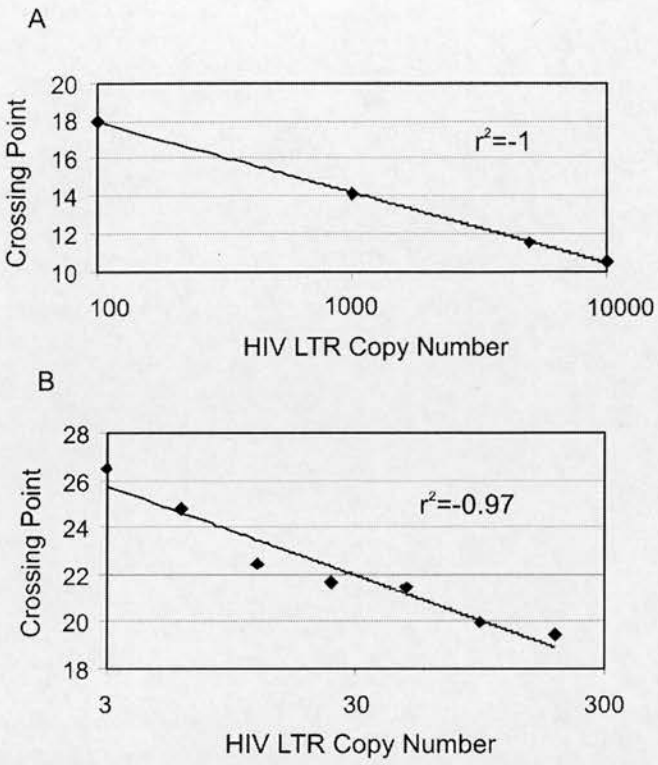


Figure 3-11. Effect of DNA concentration on LTR quantification. LTR was quantified from standard solutions containing 50, 100 and 500 LTR copies, using the nested PCR protocol described (section 2.5.2). The DNA concentration in the primary reaction was either 15 µg/ml (blue triangles), 30 µg/ml (red squares) or 90µg/ml (green diamonds). The crossing point is the number of real-time PCR cycles required to generate a given amount of template. The higher crossing points seen for reactions containing 90µg/ml DNA indicate inhibition.

The amplification efficiency of the real-time PCR was not lower in the 900μg/ml samples suggesting that the inhibition occurred during the primary reaction, this is not surprising given that the non-template DNA concentration is higher at this stage. In view of these findings a DNA concentration of up to 150μg/ml was used in test samples. As 2μl DNA was used in each reaction this allowed interrogation of 0.3μg DNA per reaction (equivalent to approximately $5x10^4$ cells), allowing infection frequencies as low as 40 copies per million cells to be reliably detected in a single reaction.

3.3.1.3 **Standard Curve.**

In order to quantify the starting concentration of target DNA using real-time PCR, the amplification efficiency must be constant throughout the exponential phase of the reaction, and must be constant between individual reactions performed in a single run. If both of these criteria are met, a plot of crossing point against logarithm of sample concentration (termed the standard curve) will generate a straight line. To test this DNA extracted from an HIV infected cell line was serially diluted in DNA extracted from HIV negative PBMCs at 100μg/ml. The standard curve generated had a linear regression coefficient of 1.00, and a standard mean error of 0.028 (Figure 3-12A). To ensure that these results were maintained for template prepared under the conditions of a test sample, the standard curve was also assessed using DNA extracted from CD4 lymphocytes isolated from an HIV infected subject using the standard protocol employed for the test samples. In this experiment the regression coefficient and error were not as good (r^2=-0.97, mean squared error = 0.22, Figure 3-12B). However, the calculated starting copy number was always within a factor of two of the true starting copy number (Figure 3-12B), and therefore the assay was considered suitable for the intended purpose.



Actual number of LTR copies	Calculated number of LTR copies
192	139
96	100
48	41
24	36
12	22
6	5
2 to 4	2

Figure 3-12. Standard curves for LTR real-time PCR. Standard curves for a serial dilution of DNA extracted from HIV infected cell line (A), and for a serial dilution of DNA extracted from CD4 lymphocytes isolated from an HIV infected subject (B). The actual number of LTR copies in each reaction, and the calculated estimates are given (B).

3.3.1.4 **External standards.**

The number of LTR copies in test samples were quantified against in house standards consisting of a serial dilution of a full length HIV clone diluted in DNA extracted from HIV –ve PBMCs at a concentration of 100μg/ml. Four dilutions, ranging from 10 LTR copies per reaction to 5000 LTR copies per reaction, were included in each test run. Use of external standards to quantify test samples is only valid if the amplification efficiency of the standards and test samples are closely matched. Amplification efficiency describes the fold amplification of target DNA per PCR cycle, and a value close to two is expected. Use of the HIV LTR as the target sequence in the standard, and dilution of standards in DNA extracted from PBMCs increased the likelihood of comparable amplification efficiencies.

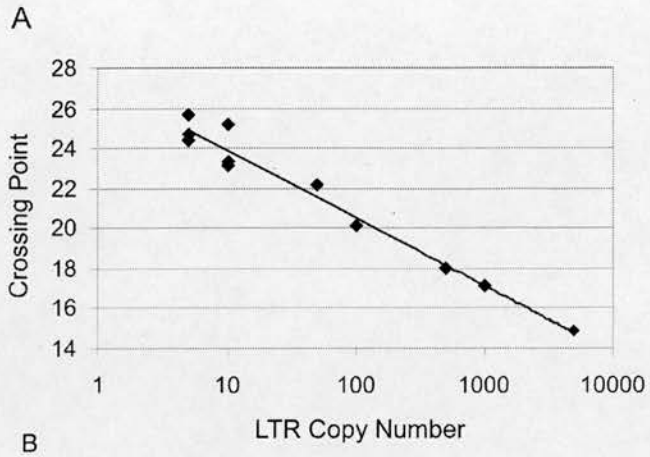
The amplification efficiency of the standards and two test samples was determined by performing the nested real-time PCR on dilution series of the standards and of the two test samples. Amplification efficiency was calculated using the equation:

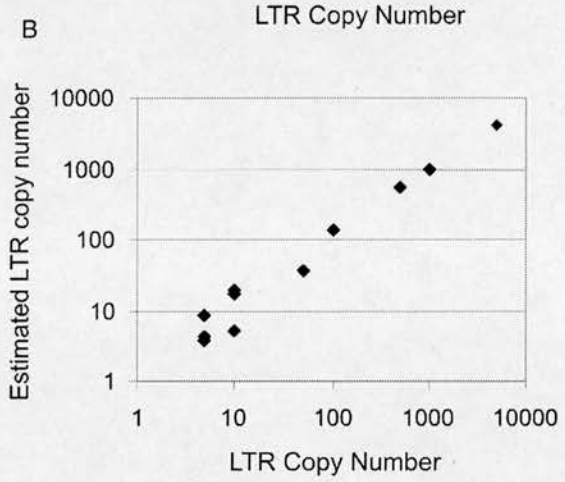
$E = 10^{-1/slope}$ where E = amplification efficiency, slope = the slope of the linear regression line through the data points on a plot of crossing point against logarithm of sample concentration.

The two test samples used were: i) a sample of DNA extracted from an HIV infected cell line, ii) DNA extracted from paraformaldehyde fixed CD4 lymphocytes isolated from an HIV infected subject (this sample was treated in exactly the same way as test samples used in the main study). The amplification efficiencies were 1.81 for the standard, 1.85 for the HIV infected CTL line and 1.84 for the cells isolated from the HIV infected subject. These amplification efficiencies differ by less than 0.05 and thus the standards were considered suitable.

3.3.1.5 **Reliability at low copy numbers.**

As the expected proviral loads in the test samples were low, it was important to ensure that the nested real-time PCR protocol remained reliable at low starting target copy numbers. To assess this a dilution series of the external standards was run with the five copy and ten copy reactions performed in triplicate. As expected deviation from the standard curve did increase with lower starting copy numbers, however the calculated copy number remained within a factor of 2.0 of the actual copy number (Figure 3-13).





LTR copy number	Estimated LTR copy number
5000	4143
1000	941
500	555
100	136
50	37
10	17
10	20
10	5
5	4
5	9
5	4

Figure 3-13. Use of LTR real-time PCR for quantification of low copy numbers. The reliability of the nested real-time PCR protocol at quantifying template at low copy number was assessed using triplicate reactions of 5 and 10 starting LTR copies. Deviation from the standard curve is seen at these low copy numbers (A), but estimated copy numbers remain within a factor of 2 of the actual copy numbers (B).

3.3.1.6 **Ongoing quality control.**

During the course of data collection, the amplification efficiency of six test samples was monitored by inclusion of two dilutions of the test sample in the quantification reaction. This monitoring demonstrated that in one of the samples there was a marked difference in the proviral loads estimated from the two dilutions suggesting either a pipetting error or marked failure of one of the amplification reactions, and in another sample more provirus was detected in the diluted than the neat sample making amplification efficiency impossible to assess. In the remaining four samples

the amplification efficiency of the test samples tended to be lower than that of the standards (mean of tests = 1.76, mean of standards = 1.96). This difference was sufficient to cause underestimation of the proviral load in the test sample by 1 log over 22 cycles (10 proviral copies were generally detected after approx 22 cycles). The under-estimation of the proviral load caused by the lower amplification efficiency is more marked for lower starting copy numbers, and thus this bias would tend to decrease the estimated viral load in CD8 populations as compared to CD4 populations. This could thus lead to underestimation, but not overestimation of the proviral load attributable to CD8 lymphocytes. In view of this observed difference in the amplification efficiency, and the relatively frequent occurrence of clearly erroneous results, it was decided to confirm all proviral loads using limiting dilution.

3.3.2 Quantification of HIV provirus by limiting dilution PCR.

Limiting dilution PCR of HIV LTR has been previously used to quantify HIV proviral load (McBreen *et al.*, 2001; Imlach *et al.*, 2001), and the method (described in section 2.5.3) was not modified for this project. Sensitivity to a single copy was confirmed using a standard dilution series (3.3.1.1). The reproducibility of the protocol for DNA extraction and limiting dilution LTR quantification was tested on five replicate samples of paraformaldehyde fixed PBMCs from an HIV infected subject, and was found to be good with the calculated proviral load ranging from 13 – 50 proviral copies per million cells. The correlation between the original real-time PCR estimate and the final result provided by limiting dilution was $r_s = 0.86$, $p<0.01$ (Figure 3-14).

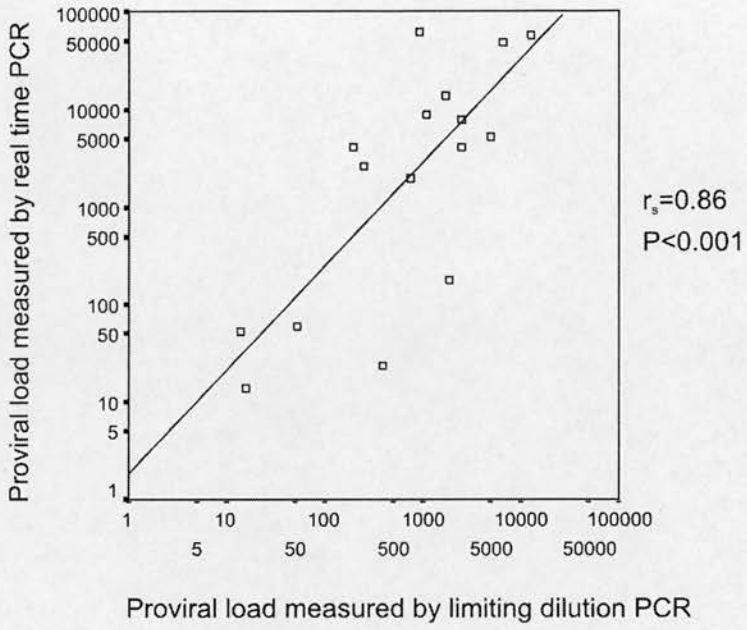


Figure 3-14. Correlation between the real-time PCR estimate and limiting dilution estimate of proviral load. r_s = Spearmans rank correlation coefficient.

3.4 Conclusion

Immunomagnetic methods alone were found to be inadequate in terms of both yield and purity for isolation of CD8 lymphocyte subsets, and therefore a two stage process of immunomagnetic enrichment followed by FACS was developed. This process allowed isolation of naïve, memory, effector, $CD8^{bright}CD4^{dim}$ and CD8+CD4- populations with high purity. The use of FACS necessitated cell fixation and conditions for fixing cells and subsequent DNA extraction were optimized. Fixation with 0.75% paraformaldehyde for one hour coupled with a DNA extraction method with an extended period of cell lysis allowed extraction of over 75% of the expected DNA. A real-time PCR method was developed to quantify HIV LTR copies. Using a nested approach infection frequencies down to 40 proviral copies per million cells could be detected in a single reaction, and quantification was accurate to within a factor of two. Ongoing monitoring of the method revealed some inconsistent results and thus all LTR copy number estimates were confirmed at

limiting dilution. There was a good correlation between the real-time and limiting dilution estimates.

Chapter 4: HIV infection of CD8 lymphocytes.

4.1 Introduction

As discussed in section 1.10, HIV infection of CD8 lymphocytes has been repeatedly demonstrated *in vitro* and, while HIV provirus has been detected in CD8 lymphocyte populations isolated from both the blood and lung of HIV infected subjects, some commentators argue that this provirus may have originated in contaminating CD4 lymphocytes.

Likely mechanisms for HIV entry into CD8 lymphocytes include CD4 dependent and CD4 independent routes, as introduced in section 1.11. The routes thought most likely to generate the majority of circulating HIV infected CD8 lymphocytes are export of intrathymically infected cells, or infection during CD4 upregulation on activation of the mature CD8 lymphocyte.

It is well known that mature T lymphocytes develop from thymocytes which pass through a stage of CD4 and CD8 co-expression, and that these double positive thymocytes then completely downregulate either CD4 or CD8 to become single positive CD8 'cytotoxic' or CD4 'helper' lymphocytes. Double positive thymocytes show marked susceptibility to productive infection by both R5 and X4 tropic HIV *in vitro,* and infected cells can then mature into $CD3^{high}CD8^{+}$ thymoctes (De Rossi *et al.*, 1990; Stanley *et al.*, 1993; Su *et al.*, 1995). The mechanism of infection is likely to be through classic CD4 mediated viral entry as $CD3^{high}CD8^{+}$ thymocytes are resistant to infection (Lee *et al.*, 1997).

Thymic atrophy is a common consequence of HIV disease in both adults and children (Grody *et al.*, 1985; Joshi *et al.*, 1986), and HIV infected cells have been demonstrated by in situ hybridisation in thymic tissue removed from HIV infected individuals (Burke *et al.*, 1995; Haynes *et al.*, 1999). These infected cells are most likely to include thymocytes, an interpretation supported by the isolation of an HIV variant with higher than normal replicative capacity in thymic cell lines (Calabro *et*

al., 1995), but definitive co-localisation of HIV and thymocyte markers is lacking. In the thy/hu mouse HIV infected thymocytes can be exported into the circulation (Brooks *et al.*, 2001), but whether the thymus is the source of the circulating CD8 infected lymphocytes observed in HIV infected individuals remains unclear.

As described in section 1.5.3, *in vitro* stimulation of CD8 lymphocytes leads to upregulation of CD4 and generation of cells with a $CD8^{bright}CD4^{dim}$ phenotype. These $CD8^{bright}CD4^{dim}$ lymphocytes are susceptible to productive HIV infection, and this infection is CD4 dependent (Flamand *et al.*, 1998; Kitchen *et al.*, 1998; Zhang *et al.*, 2001; Zloza *et al.*, 2003).

Two studies have investigated the likely source of HIV infected CD8 lymphocytes circulating *in vivo*, by assessment of the relative frequency of HIV infection of CD8 lymphocyte subpopulations isolated from HIV infected subjects. In the first study a higher frequency of HIV infected cells was found in antigen naïve than antigen experienced cells, supporting a thymic source (McBreen *et al.*, 2001). However CD45RA expression was used as the sole marker of naïve status, and it was subsequent discovered that this molecule is expressed on effector as well as naïve CD8 lymphocytes (Hamann *et al.*, 1997). In the second study CD8 lymphocytes were divided on the basis of CD4 co-expression. Frequency of infection was much greater in $CD8^{+}CD4^{+}$ than $CD8^{+}CD4^{-}$ lymphocytes, suggesting direct infection of this circulating double positive population rather than intrathymic infection (Imlach *et al.*, 2001). Subpopulations were isolated by immunomagnetic technology therefore it was not possible to differentiate $CD8^{bright}CD4^{dim}$ from $CD4^{bright}CD8^{dim}$ lymphocytes. More recently Brenchley *et al.*, (2004a) demonstrated that HIV proviral load in $CD8^{+}CD4^{dim}$ lymphocytes was 5 to 100 times that found in memory CD8 lymphocytes not expressing CD4.

To confirm HIV infection of CD8 lymphocytes *in vivo,* and demonstrate that observed provirus does not originate from contaminating cells, HIV provirus was quantified from highly purified CD8 lymphocyte populations. To investigate the mechanism of infection, HIV proviral load was assessed in CD8 lymphocyte subsets isolated from the blood of 20 HIV infected subjects. The rationale behind the subsets isolated is given in section 3.1.

Primary HIV infection (PHI) can be defined as the period between HIV infection of a subject and the establishment of the viral load set point. Events during this period have a major influence on the course of disease, with poor viraemic control being prognostic of rapid progression (Lyles *et al.*, 2000). CD8 lymphocytes are vital in the control of initial viraemia (Koup *et al.*, 1994; Musey *et al.*, 1997) and thus any compromise of CD8 lymphocyte function at this stage could have a profound effect on prognosis. To investigate whether significant infection of CD8 lymphocytes occurs in PHI, the level of HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes was determined in a single subject during PHI.

4.2 Subjects and Samples

Nineteen subjects with chronic HIV infection, and one with PHI, attending health care services in Scotland were recruited with each subject giving informed consent for participation in the study in accordance with Lothian Regional Ethics Committee (LREC2001/4/25). The only inclusion criteria for recruitment was HIV infection. Exclusion criteria included anaemia (Hb < 100 g/L) or involvement in a clinical trial. Data collected for each subject included age, most recent CD4 count and viral load, and antiretroviral treatment history. The CD4 count was determined in the Western General Hospital haematology laboratory by flow cytometry using MultiTEST IMK Kits and TruCOUNT tubes (Becton Dickenson). Viral load was determined in the Regional Virus Laboratory, Edinburgh Royal Infirmary, using a quantitive PCR (Roche), with sensitivity to 50 copies/ml blood. The clinician attending the subject was asked to indicate whether the subject had any intercurrent infection on the day that blood was drawn for the study.

The subjects had a mean age of 37, and were at various stages of disease progression from asymptomatic through to AIDS. Three subjects were long term non-progressors (defined as subjects who had maintained a CD4 count above 300 without antiretroviral therapy despite over 10 years of infection), and thirteen were receiving combination antiretroviral therapy at the time of sampling (Table 4-1).

A 30 – 50ml blood sample was drawn from each subject. As each sample contained insufficient CD8 lymphocytes to assess HIV infection of all the cell subsets of interest, the samples were divided into two groups. CD8 lymphocytes from group 1 samples were divided on the basis of CD4 expression (N=9), and those from group 2 on the basis of differentiation phenotype (N=11). One subject (subject 19) donated a blood sample on two occasions and is therefore included in both groups. Subjects were selected to ensure a range of disease stages from asymptomatic to advanced AIDS were represented in each group.

The subject with primary HIV infection presented with a typical HIV seroconversion illness consisting of fever, rash and lymphadenopathy approximately four weeks after HIV exposure through unprotected heterosexual intercourse. Primary HIV infection was confirmed by two weakly reactive HIV antibody tests with a negative western blot (4^{th} generation HIV combo assay, Abbott Diagnostics; 4^{th} generation Vidas Duo assay, BioMerieux; and New LAV Blot 1, BioRad). HIVp24 antigen was positive (Vidas p24 antigen assay, BioMerieux) and could be neutralised with specific antiserum. The initial HIV viral load was $3.5x10^6$ RNA copies/ml serum (Roche Cobas Monitor HIV RNA assay, Roche Diagnostics) rising to $6.3 x10^6$ a week later, then dropping to $2x10^4$ after one month without therapeutic intervention. These tests were performed in the Regional Virus Laboratory, Gartnavel General Hospital, Glasgow.

Study subject[a]	Study group[b]	CD4 lymphocyte count / µl blood	Plasma viral load/ml	Combination antiretroviral[c]	Recent or ongoing acute infection	Age
1	1	2	75,000	No	Pulmonary tuberculosis	47
2	2	17	750,000	Yes	Pyrexia of unknown origin	29
3	2	39	309	Yes	None	36
4	2	51	23,100	No	Pneumonia	43
5	1	66	75,000	Yes	Clinical diagnosis of PML, JC virus not isolated	44
6	1	117	75,000	Yes	None	43
7	1	123	330,000	Yes	Oral candidiasis	31
8	2	296	470	Yes	None	32
9	2	328	<50	Yes		33
10	2	334	<50	No	None	43
11	1	339	14,900	Yes	None	34
12*	2	364	304	No	None	37
13	1	385	153	Yes	None	37
14	1	393	403	Yes	None	40
15*	1	396	862	No	Cellulitis	37
16	2	509	<50	Yes	None	54
17	2	531	<50	Yes	Small bowel overgrowth	38
18*	2	647	13400	No	None	38
19A	2	824	1900	Yes	None	40
19B	1	887	<50	Yes	None	41
20	1	630	3,500,000	No	None	34

Table 4-1. Subject characteristics. Subjects ordered in ascending CD4 lymphocyte count except subject 20 who presented with primary HIV infection. a: subjects indicated by * are long term non-progressors. b: CD8 lymphocytes from samples in study group 1 were subdivided on the basis of CD4 expression, those in study group 2 were subdivided by differentiation phenotype. c: Yes indicates subject was prescribed at least three antiretrovirals at the time the sample was taken.

4.3 Results.

The results are presented in two sections. In section 4.3.1 the characteristics of the isolated lymphocyte populations in terms of cell numbers and purity are provided. The purity data is important as it is used to demonstrate that the HIV detected in the CD8 lymphocyte populations originated in the CD8 lymphocytes not contaminating cells. The overall CD8 lymphocyte proviral load and the distribution of provirus within the CD8 lymphocyte subsets are presented in section 4.3.2.

4.3.1 Isolated CD8 lymphocyte populations.

CD8 lymphocytes were isolated using a two step protocol involving initial immunomagnetic enrichment followed by FACS (Figure 2-1, see section 2.1 for details of protocols and section 3.1 for development of cell isolation methods). CD8 lymphocytes from group 1 samples were divided into those expressing CD4 ($CD8^{bright}CD4^{dim}$) and those not expressing CD4 ($CD8^{bright}CD4^{-}$). CD8 lymphocytes from group 2 samples were divided into antigen naïve (CD45RA+CD27high) and antigen experienced (all non-antigen naïve CD8 lymphocytes), and in 7 samples the antigen experienced population was further subdivided into memory (CD45RA-CD27+) and effector (CD45RA+CD27-) subsets.

4.3.1.1 **Size of CD8 lymphocyte populations isolated.**

The number of cells isolated in each CD8 lymphocyte subset had a bearing on the availability of cells for purity assessment and HIV provirus detection. A minimum of 10^6 total CD8 lymphocytes was isolated from each subject, sufficient to quantify HIV proviral loads down to approximately 10 copies per million CD8 lymphocytes (lower proviral loads could be detected but could not be confidently ascribed to CD8 lymphocytes, see section 4.4.1.2). Cell numbers isolated in each CD8 lymphocyte subset varied widely (Table 4-2), dropping to a mean of $7.0x10^4$ for $CD8^{bright}CD4^{dim}$ lymphocytes. The low cell numbers available in this subset meant that levels of HIV infection of less than 100 copies per million cells were unlikely to be detected.

CD8 lymphocyte subset	Cells isolated per subject		
	Mean	Minimum	Maximum
All CD8 lymphocytes	$7.1x10^6$	$1.0x10^6$	$1.6x10^7$
$CD8^{bright}CD4^{dim}$ lymphocytes	$7x10^4$	$8x10^3$	$3x10^5$
$CD8^{bright}CD4^-$ lymphocytes	$5.6x10^6$	$1.0x10^6$	$1.4x10^7$
Naïve CD8 lymphocytes	$1.1x10^6$	$3x10^5$	$3x10^6$
Antigen experienced CD8 lymphocytes	$6.8x10^6$	$1.1x10^6$	$1.4x10^7$
Memory CD8 lymphocytes	$1.1x10^6$	$1.7x10^5$	$2.4x10^6$
Effector CD8 lymphocytes	$1.0x10^6$	$6x10^4$	$1.8x10^6$

Table 4-2. CD8 lymphocyte subset yield. The mean and range of the number of cells isolated in each CD8 lymphocyte subset across the 20 samples from subjects with chronic HIV infection.

4.3.1.2 **Purity of isolated CD8 lymphocyte subsets.**

Assessment of the level of contamination of CD8 lymphocyte subsets with other potentially HIV infected cells was vital for calculation of the CD8 lymphocyte proviral load. Thus, isolated CD8 lymphocyte subpopulations were assessed by flow cytometry to determine the level of contamination with: a) CD4 lymphocytes, b) any non-CD8 lymphocyte cell, and c) CD8 lymphocytes of the incorrect subtype. The number of cells acquired in each purity assessment varied according to the number of cells available (Table 4-). Unless otherwise stated the values given are ungated, ie. all events acquired by the flow cytometer are included in the analysis. This is the most stringent measure of purity. For some indices 'gated' values are given in which events likely to represent non-cellular events such as bubbles, or cell debris are excluded from the analysis (Figure 4-1). While less stringent, these values are likely to more closely represent the actual purity of the isolated cells.

a) CD4 lymphocyte contamination, defined as the percentage of the total analysed events that displayed a CD8β-CD4+ phenotype, was determined for all CD8

lymphocyte populations where cell numbers allowed, and was found to be low with an overall median level of 0.10% (Table 4-). Gated and ungated data are provided for group 2 samples. For group 1 samples only gated data are provided, this is due to the high number of non-cellular events which displayed the same fluorescence as CD8β-CD4+ cells (Figure 4-1). The ungated values for group 2 and gated values for group 1 were then used to calculate the 'best estimate' proviral load attributable to CD8 lymphocytes (section 2.5.4).

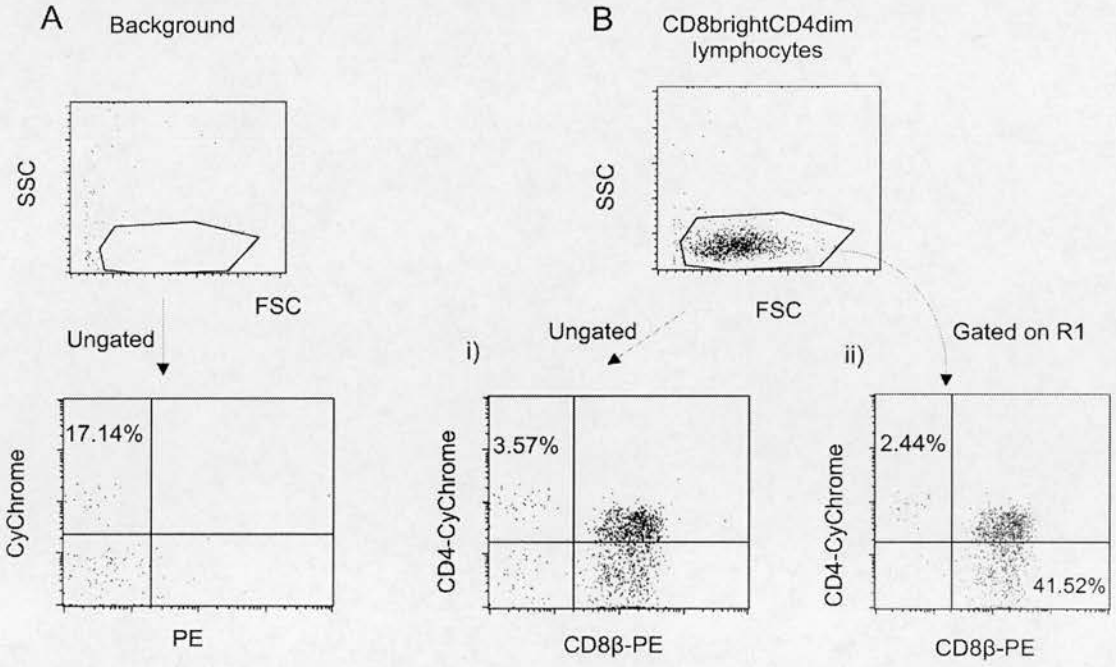


Figure 4-1. Background events influencing $CD8^{bright}CD4^{dim}$ purity assessment. (A) Background events acquired on running FACS acquisition fluid for 1 minute. The fsc against ssc plot shows that few background events fall within the lymphocyte gate. The PE against CyChrome plot shows that 17.14% of background events mimic CD4 lymphocytes ($CyChrome^{+}PE^{-}$). (B) $CD8^{bright}CD4^{dim}$ lymphocytes purity check (data from sample 14). i) ungated analysis: the $CD4^{+}CD8\beta^{-}$ quadrant includes both true contaminating CD4 lymphocytes and background events, giving a falsely high level of CD4 lymphocyte contamination. ii) gated on live lymphocytyes (R1): a more accurate level of CD4 lymphocyte contamination is apparent. (This subject shows the highest level of CD4 lymphocyte contamination seen in this study, accounting for 43 proviral copies per million $CD8^{bright}CD4^{dim}$ lymphocytes). Almost half the isolated $CD8^{bright}CD4^{dim}$ population appears to have a CD4-ve phenotype, this is due to fluorochrome bleaching during the sorting process.

b) As HIV infection of CD4 lymphocytes is known to down-regulate surface CD4 expression, it could be argued that defining CD4 lymphocytes as cells expressing CD4 could underestimate the true level of CD4 lymphocyte contamination. To allow for this possibility, and provide a 'worst case' measure of contamination, the overall level of contamination of CD8 lymphocyte populations with non-CD8 lymphocytes was measured. This value, defined as the percentage of total analysed events with a CD8β-ve phenotype, was also found to be low, with an overall median for all CD8 lymphocyte subsets of 0.97% (0.34% gated) (Table 4-) . Gates defining CD8β expression were set using the presort sample (Figure 4-2).

c) Finally, to assess whether HIV provirus found in less commonly infected CD8 lymphocyte subsets could be attributed to contamination with cells from the more commonly infected subset, the level of contamination of CD8+CD4- lymphocytes with $CD8^{bright}CD4^{dim}$ lymphocytes and of naïve CD8 lymphocytes with antigen experienced CD8 lymphocytes was estimated. For the purpose of this assessment all cells with low expression of either CD45RA or CD27 were defined as antigen experienced, with gates set on the pre-sort sample (Figure 4-2). Levels of contamination were higher than noted for CD4 lymphocyte contamination (Table 4-), and the implication of these results in the interpretation of provirus observed in CD8+CD4- lymphocytes and naïve CD8 lymphocytes are discussed in section 4.4.1.1.

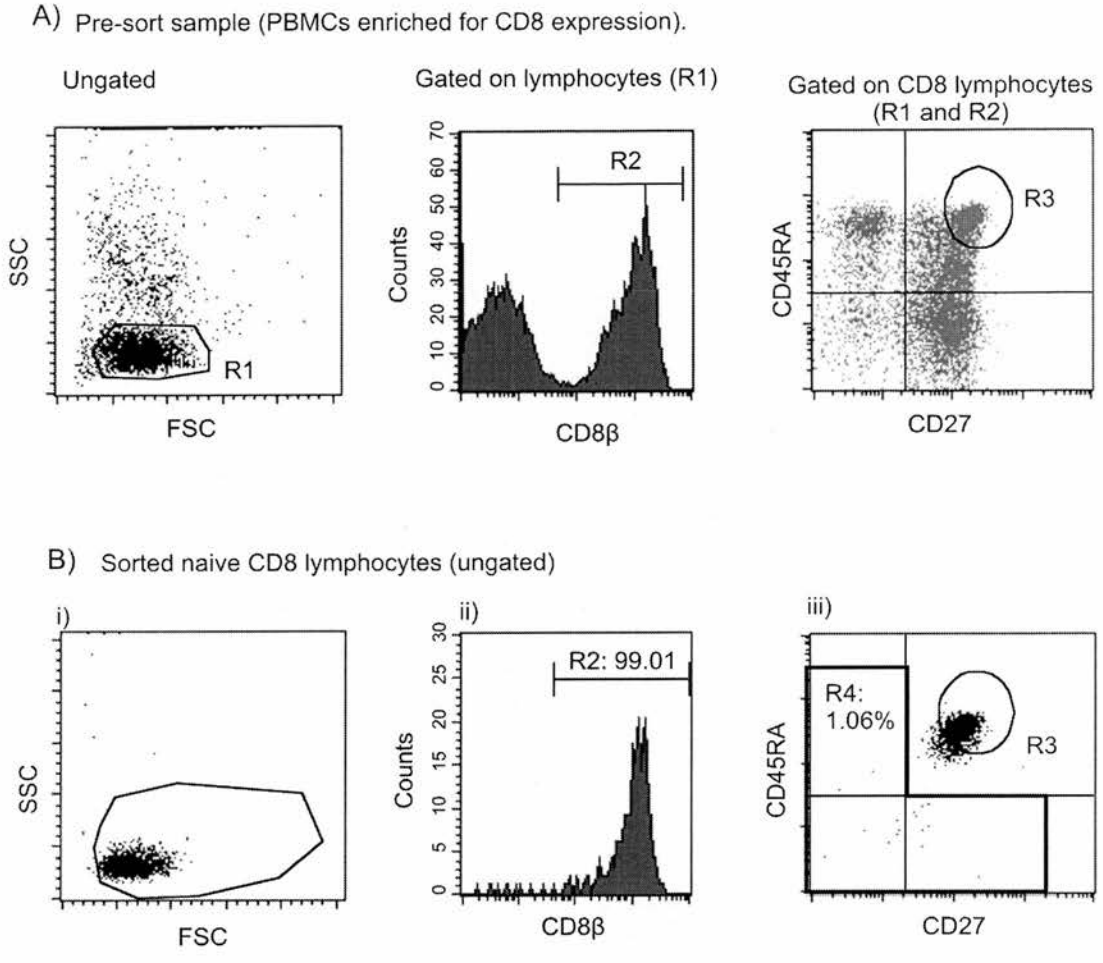


Figure 4-2. Method of gating for assessment of contamination of naïve CD8 lymphocytes with non-CD8 lymphocytes and experienced CD8 lymphocytes. A) Gates for isolation of naïve CD8 lymphocytes were set using the pre-sort sample, typical positions are shown. B) Gates for purity assessment: i) A wide fsc ssc gate was used to generate gated and ungated purity data so as to include all events representing lymphocytes in the gated population. ii) The gate R2 set on the presort sample was used to assess contamination with CD8β-ve cells. iii) The gate R3 used to define naïve cells was not appropriate for purity assessment due to fluorochrome bleaching, therefore contaminating CD8 experienced cells were defined as CD45RA-ve or CD27-ve (gate R4).

Subject	% CD4 lymphocyte contamination of populations. (gated data only)		% non-CD8 lymphocyte contamination of populations. (gated and ungated data given)				% $CD8^{bright}CD4^{dim}$ lymphocyte contamination of CD8+CD4- population[a], (gated and ungated data given)	
	CD8+CD4- lymphocytes A mean of 31554 (minimum 870) cells were analysed for each subject.	**$CD8^{bright}CD4^{dim}$ lymphocytes** A mean of 804 (minimum 135) cells were analysed for each subject.	**CD8+CD4- lymphocytes** A mean of 31554 (minimum 870) cells were analysed for each subject.		**$CD8^{bright}CD4^{dim}$ lymphocytes** A mean of 804 (minimum 135) cells were analysed for each subject.			
	Gated	Gated	No gate	Gated	No gate	Gated	No gate	Gated
1	$<5x10^{-3}$	na	0.9	0.07	na	na		
5	$<9x10^{-4}$	na	0.42	0.17	na	na		
6	0.1	0.56	1.59	0.6	4.87	1.21		
7	0.01	na	1.52	0.16	na	na		
11	0.17	0.20	0.9	0.18	5.02	0.2		
13	0.01	na	1.24	0.64	na	na	1.44	1.06
14	0.13	2.44	0.76	0.52	7.42	2.99	0.37	0.24
15	0.3	na	1.52	0.9	na	na		
19B	<0.11	<0.74	1.15	0.69	0	0	0.11	0.11
20 (PHI)	na	0.3	na	na	na	0.9		
Mean			**1.1**	**0.4**	**4.3**	**1.1**	**0.6**	**0.5**
Median	**0.1**	**0.3**	**1.15**	**0.52**	**4.94**	**0.7**	**0.4**	**0.2**

Table 4-3A. The purity of CD8 lymphocyte subsets, group 1 samples. Purity was assessed by flow cytometry on a sample taken from the isolated population. CD4 lymphocytes were defined as all CD4+CD8- events. Non-CD8 lymphocytes were defined as all CD8β-ve events. Gated data was restricted to events with forward and side light scatter properties of live cells. a: data only given for CD8+CD4- lymphocyte samples in which HIV provirus was detected. na = not available. PHI = primary HIV infection.

Subject	% CD4 lymphocyte contamination of population. (ungated data only)		% non-CD8 lymphocyte contamination of population (gated and ungated values given).				% antigen experienced CD8 lymphocyte contamination of naïve CD8 lymphocytes. (gated and ungated values given). A mean of 1192 (minimum 480) cells were analysed for each subject.	
	Naïve CD8 lymphocytes A mean of 4157 (minimum 1020) cells were analysed for each subject.	**Antigen Experienced CD8 lymphocytes.** A mean of 5965 (minimum 2025) cells were analysed for each subject.	**Naïve CD8 lymphocytes.** A mean of 1192 (minimum 480) cells were analysed for each subject.		**Antigen Experienced CD8 lymphocytes** A mean of 6486 (minimum 585) cells were analysed for each subject.			
	No gate	No gate	No gate	Gated	No gate	Gated	No gate	Gated
2	0.2	<0.01	1.37	0.35	0.78	0.6	4.1	na
3	<0.03	na	0.97	0.19	0.22	0.08	na	na
4	<0.03	0.1	2.71	1.27	4.79	1.96	2.61	1.82
8	<0.03	0.03	0.97	0.5	0.08	0.08	4.64	na
9	na	na	0.8	0.35	0.18	0.12	1.84	0.84
10	0.41	na	0.99	0.36	0.35	0.15	1.06	0.57
12	<0.05	<0.05	2.13	1.02	na	na	2.27	1.1
16	0.04	0.03	0.9	0.52	0.18	0.09	3.59	3.31
17	na	na	1.31	1.21	0	0	2.8	2.59
18	0.1	0.79	0.54	0.23	0.16	0.12	4.26	3.5
19A	0.02	0.04	1.34	0.5	1.53	0.91	3.64	3.97
Mean			**1.28**	**0.59**	**0.83**	**0.41**	**3.1**	**2.2**
Median	**0.04**	**0.03**	**0.99**	**0.5**	**0.2**	**0.12**	**3.2**	**2.2**

Table 4-3B. The purity of CD8 lymphocyte subsets, group 2 samples. Purity was assessed by flow cytometry on a sample taken from the isolated population. CD4 lymphocytes were defined as all CD4+CD8- events. Non-CD8 lymphocytes were defined as all CD8β-ve events. Gated data was restricted to events with forward and side light scatter properties of live cells. na = not available.

4.3.2 HIV proviral load in CD8 lymphocytes.

The HIV proviral load in each lymphocyte population isolated was determined by DNA extraction followed by quantative PCR for HIV LTR (method given in section 2.5). A 'best estimate' HIV proviral load attributable to each CD8 lymphocyte population was determined by subtracting the provirus attributable to measured CD4 lymphocyte contamination (2.5.4). Ungated values of CD4 lymphocyte contamination were used for group 2 samples and gated values for group 1 samples (section 4.3.1.2). In addition a 'worst case scenario' HIV proviral load was calculated by assuming that all non-CD8 lymphocytes in the isolated populations had the same proviral load as CD4 lymphocytes. Gated values for non-CD8 lymphocyte contamination were used.

4.3.2.1 **HIV proviral loads in CD8 lymphocytes in chronic HIV infection**

HIV infection of CD8 lymphocytes was demonstrated in 16 out of 19 subjects using the 'best estimate' method (Table 4-4, Figure 4-3). In 12 of these 16 subjects the HIV proviral load attributable to CD8 lymphocytes was more than ten times that attributable to contaminating CD4 lymphocytes (Figure 4-4). The level of infection was low (median 7 HIV DNA copies per million CD8 lymphocytes, range undetectable – 218; Figure 4-3), and was significantly lower than that in CD4 lymphocytes from the same subjects (median 1176 HIV DNA copies / million cells, range 8 to 12756, $P<0.001$, Wilcoxon Signed Rank Test; Figure 4-3A). No significant correlation was found between HIV DNA loads in CD4 and CD8 lymphocytes, (Spearmans correlation coefficient 0.3, $p>0.1$).

Using the 'worst case scenario' contamination data HIV infection of CD8 lymphocytes was demonstrated in 13 out of 19 subjects, with 7 of these having over 10 times more provirus attributable to CD8 lymphocytes than contaminating cells (Table 4-4).

Table 4-4b. HIV proviral loads for lymphocyte subsets of group 2 samples.

Subject Characteristics				Attributable HIV DNA copies per million cells (bold), standard error in parenthesis, proviral load attributable to CD4 lymphocytes contaminating CD8 populations in italics[b]								
Study subject	CD4 count /µl	Plasma viral load/ml	Combination antiretroviral[a]	CD4 lymphocytes	Best estimated attributable CD8 lymphocyte proviral load[c]					'Worst case scenario' attributable CD8 lymphocyte proviral load[d]		
					CD8 lymphocytes	Naïve CD8 lymphocytes	Experienced CD8 lymphocytes	Memory CD8 lymphocytes	Effector CD8 lymphocytes	CD8 lymphocytes	Naïve CD8 lymphocytes	Experienced CD8 lymphocytes
2	17	750000	Yes	**8514** (3595)	**99** *1*	**ND <43** *7*	**110** *1*	**28**	**50**	**54** *49*	**ND <43** *30*	**59** *51*
3[e]	39	309	Yes	**933** (270)	**218** *0*	NI	NI	NI	NI	**217** *2*		
4	51	23100	No	**10311** (7446)	**48** *9*	**35** *3*	**50** (14) *10*	NI	NI	**ND** *193*	**ND** *131*	**ND** *202*
8	296	470	Yes	**4683** (2897)	**13** *1*	**ND <18** *1*	**15** (8) *1*	**25**	**ND <17**	**11** *na*	**ND <18** *na*	**12** (8) *4*
9	328	<50	Yes	**942** (276)	**8** *2*	**ND <65** *0*	**9** *2*	**ND <10**	**ND <22**	**9** *1*	**ND <65** *3*	**10** *1*
10	334	<50	No	**732** (402)	**14** *3*	**17** *3*	**13** (7) *3*	NI	NI	**15** *1*	**17** *3*	**15** (7) *1*
12	364	153	No	**64**	**ND <12**	**ND <10** *0*	**ND <14** *0*	**ND <22**	**ND <23**	**ND** *0*	**ND <10** *1*	**ND <14**
16	509	<50	Yes	**1320** (755)	**4** *0*	**ND <3**	**5** *0*	**11**	**ND <3**	**3** *2*	**ND <3** *7*	**4** *1*
17	531	<50	Yes	**12756** (5838)	**ND <5**	**ND <6** *4*	**ND <5** *26*	**ND <4**	**ND <37**	**ND** *40*	**ND <6** *154*	**ND <5** *0*
18	647	13400	No	**1692** (768)	**1** *0*	**6** *2*	**ND <6** *13*	**ND <17**	**ND <5**	**1** *2*	**4** *4*	**ND <6** *2*
19A	824	1900	Yes	**1031** (480)	**3** *0*	**ND <214** *0*	**4** *0*	NI	NI	**ND** *4*	**ND <214** *5*	**ND** *9*

a: Subject prescribed at least three antiretrovirals at time sample was drawn. b: Where purity data was available for experienced but not naïve subsets (subjects 3 and 10), purity of the naïve subset was assumed to be the same as that of the experienced subset. Where no purity data was available (subjects 9 and 17) a level of 0.2% was used which represents the 75th percentile of the available CD4 lymphocyte contamination data. c: Best estimate attributable CD8 proviral load calculated by subtracting provirus attributed to measured CD4 lymphocyte contamination. d: Worst case scenario attributable CD8 proviral load was calculated by subtracting provirus attributed to all non CD8 lymphocyte contamination. e: No subdivision of CD8 lymphocytes due to abnormal distribution of CD45RA and CD27 expression. ND = not detectable, NI = not isolated.

Table 4.4a. HIV proviral loads for lymphocyte subsets of group 1 samples and subject with primary HIV infection.

Subject Characteristics				Attributable HIV DNA copies per million cells (bold), standard error in parenthesis, proviral load attributable to CD4 lymphocytes contaminating CD8 populations in italics.						
Study subject	CD4 count /µl	Plasma viral load/ml	Combination antiretroviral[a]	CD4 lymphocytes	Best estimated attributable CD8 lymphocyte proviral load[b]			'Worst case scenario' attributable CD8 lymphocyte proviral load[c]		
					CD8 lymphocytes	$CD8^{bright}CD4^{dim}$ lymphocytes	CD8+CD4- lymphocytes	CD8 lymphocytes	$CD8^{bright}CD4^{dim}$ lymphocytes	CD8+CD4- lymphocytes
1	2	75000	No	**652**[d]	**5**[d] *0*	**2683**[d] *0*	ND[d] *0*	**5**[d] *0*	**2664**[d] *20*	**ND**[d] *0*
5	66	>750000	Yes	**3282** (2055)	**24** (9) *0*	**1197** (461) *0*	ND <20 *0*	**22** *0*	**1099** *98*	**ND <20** *6*
6	117	75,000	yes	**12217**	**7** *1*	**865** (533) *68*	ND <2 *12*	**6** *1*	**785** *148*	**ND <2** *73*
7	123	330000	Yes	**12608** (7835)	**79** (30) *0*	**3951** (1518) *1*	ND <41 *1*	**72** *8*	**3575** *377*	**ND <41** *20*
11	339	14900	yes	**605**	**3** *0*	**195** *1*	ND <52 *1*	**3** *0*	**195** *1*	**ND <52** *1*
13	385	153	Yes	**1830** (1295)	**2** (1.4) *3*	**48** (33) *3*	**5** *3*	**ND**	**ND** *55*	**ND** *12*
14	393	403	yes	**1897**[d]	**12**[d] *3*	**1936**[d] *46*	**7**[d] *3*	**6**[d] *0*	**1925**[d] *57*	**ND** *10*
15	396	862	No	**8**	**ND <3**	**ND <258** *0*	ND <3 *0*	**ND**	**ND** *0*	**ND <3** *0*
19B	887	<50	Yes	**254**	**2** *0*	**ND <39** *2*	**2** *0*	**ND**	**ND** *0*	**ND** *2*
20	630	3,500,000	No	**697**	**4** *0*	**523** (160) *2*	**ND <4**	**4** *0*	**519** (160) *6*	**ND <4**

a: Subject prescribed at least three antiretrovirals at time sample was drawn. b: Best estimate attributable CD8 proviral load calculated by subtracting provirus attributed to measured CD4 lymphocyte contamination. Where no purity data was available for $CD8^{bright}CD4^{dim}$ populations (subjects 1,5,7,13,15) CD4 lymphocyte contamination was assumed to be the same as that of the CD8+CD4- lymphocyte population. c: Worst case scenario attributable CD8 proviral load was calculated by subtracting provirus attributed to all non CD8 lymphocyte contamination. Where no purity data was available for $CD8^{bright}CD4^{dim}$ populations (subjects 1,5,7,13,15) a level of 2.44% was used which represents the worst recorded contamination of this cell type. d: Proviral load estimated using real-time PCR. ND = not detectable.

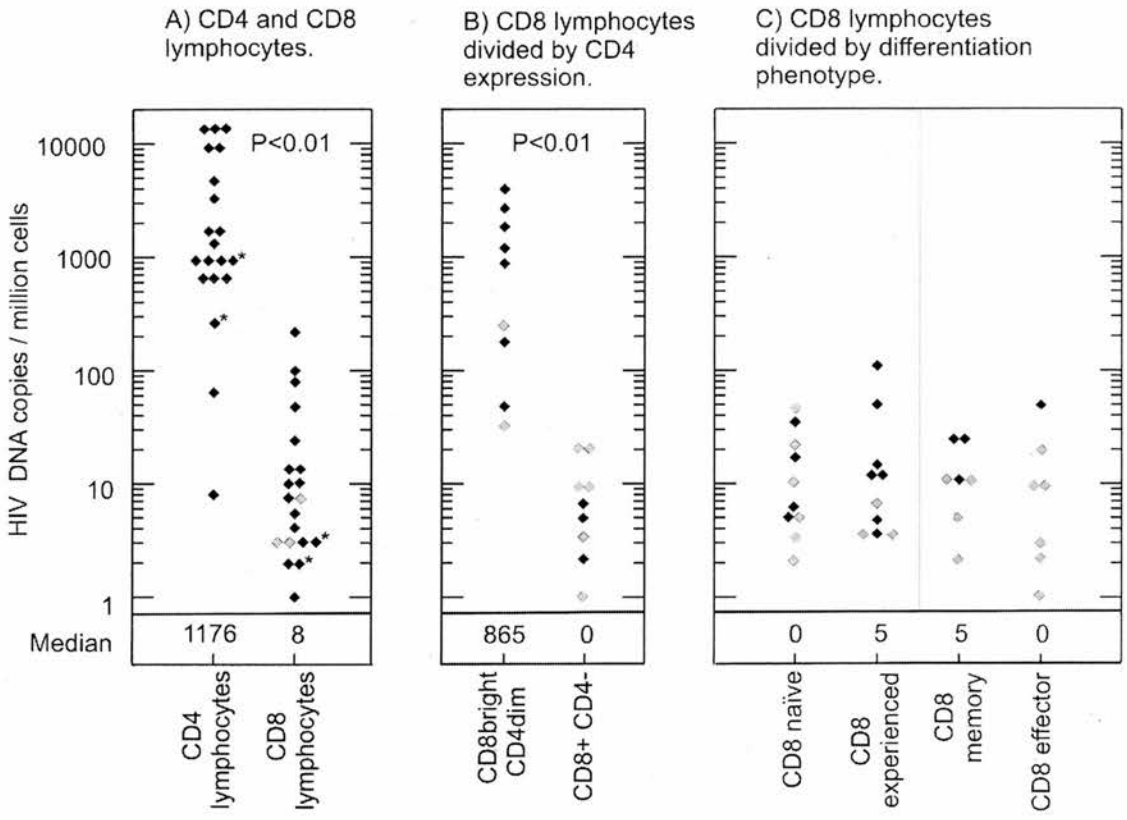


Figure 4-3. Distribution of HIV proviral loads in lymphocyte subsets. 'Best estimate' attributable proviral loads are shown. Black diamonds indicate detectable proviral load. Grey diamonds indicate samples where no virus was detected, (the value given is half the lower limit of detection which varies with the number of cells available for analysis). P values shown are calculated using the Wilcoxon Signed Ranks Test. (A) Proviral loads for CD4 and CD8 lymphocytes from all samples (group A and group B). Subject 19 was sampled twice and data points from this subject are indicated with *. (B) Proviral loads for $CD8\beta^{bright}CD4^{dim}$ and $CD8\beta^{+}CD4^{-}$ lymphocytes. (C) Proviral loads for CD8 lymphocytes divided by differentiation phenotype.

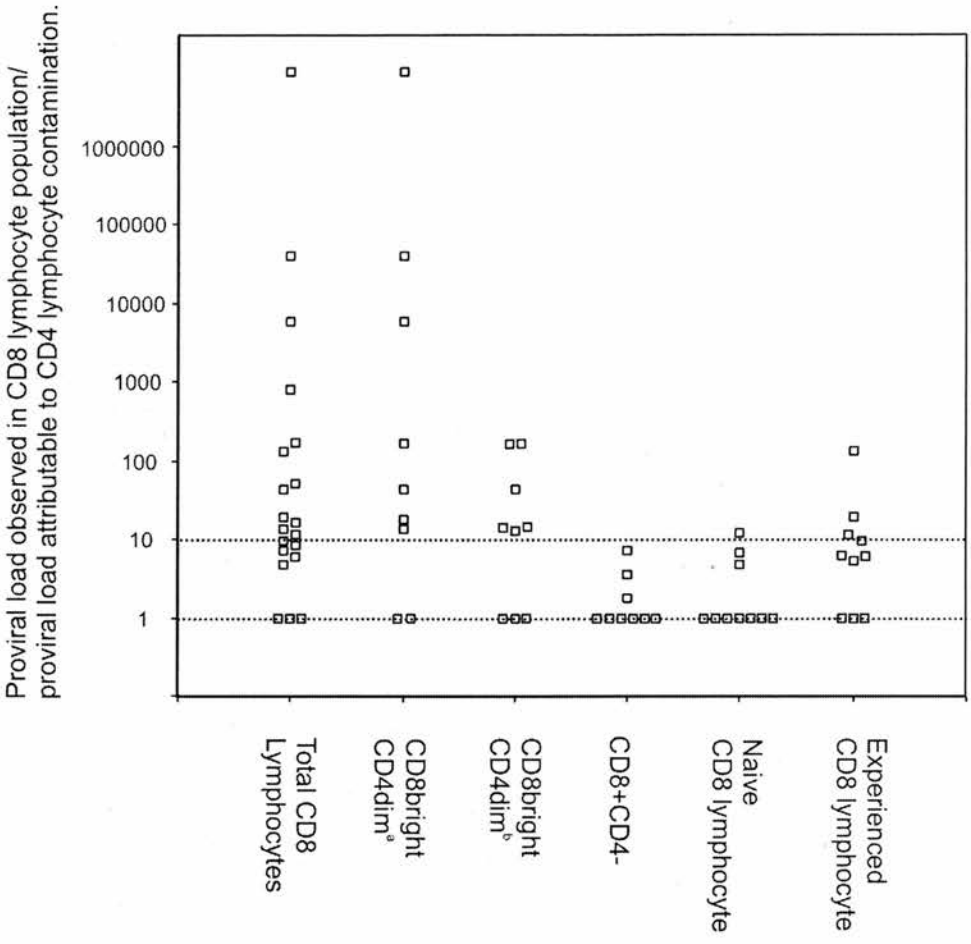


Figure 4-4. Ratio of HIV proviral load in CD8 lymphocyte populations to that attributable to CD4 lymphocyte contamination. Where the ratio is greater than 10 it is unlikely that the viral load attributed to CD8 lymphocytes could be an artefact of inaccuracies in the measurement of cell population purity or proviral load. Samples where no provirus attributed to CD8 lymhocytes were detected are given a ratio of 1. a) Where no purity data available CD4 lymphocyte contamination assumed to be the same as for $CD8^{bright}CD4^{-}$ population from the same sort; b) where no purity data available CD4 lymphocyte contamination assumed to be 2.44%, the worst level of contamination measured in the series of test samples.

4.3.2.2 Correlation of HIV infection of CD8 lymphocytes with disease progression.

There was an inverse correlation between CD4 lymphocyte count and 'best estimate' CD8 lymphocyte HIV DNA load (Spearmans correlation coefficient R=-0.78, $p<0.001$; Figure 4-5A), indicating a progressive increase in prevalence of infected

CD8 lymphocytes with advancing disease. In subjects with advanced disease the higher frequency of infected CD8 lymphocytes together with dwindling CD4 lymphocyte numbers, increased the contribution of CD8 lymphocytes to the total circulating HIV DNA load. Thus, in the subjects with the lowest CD4 lymphocyte counts, CD8 lymphocytes contributed over 25% of the total circulating lymphocyte HIV DNA load (Figure 4-5). In contrast the CD8 lymphocytes of long term non-progressors appear relatively resistant to infection with virtually no LTR copies detected.

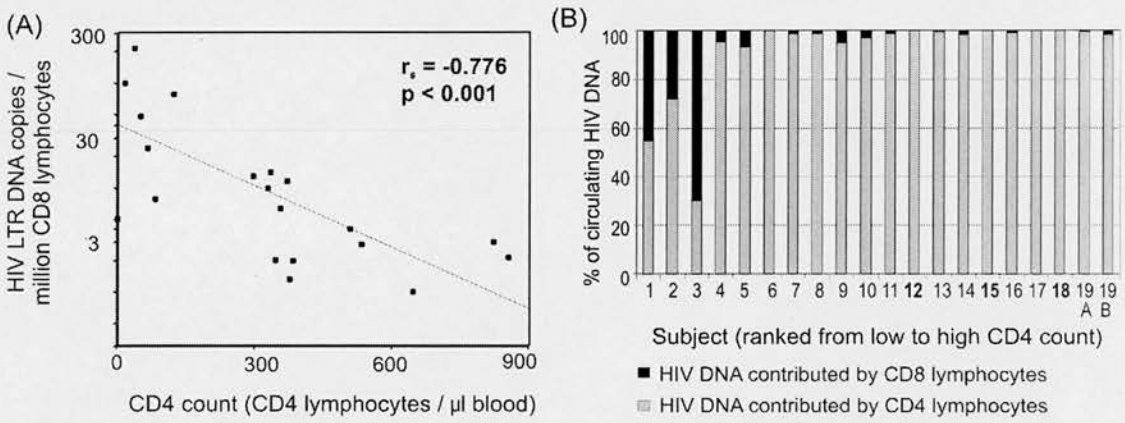


Figure 4-5. Increased HIV infection of CD8 lymphocytes with disease progression. (A) The correlation between CD8 lymphocyte HIV DNA load and CD4 count, r_s = Spearman's Rank Correlation Coefficient. Cases with undetectable proviral load were given a value of half the lower limit of detection. (B) The relative contribution of CD4 and CD8 lymphocytes to overall circulating HIV DNA load was calculated from CD4 and CD8 lymphocyte HIV DNA loads and CD4 and CD8 lymphocyte counts. Undetectable viral loads were given a value of zero. Long term non-progressors shown in bold.

4.3.2.3 **Mechanism of HIV infection of CD8 lymphocytes *in vivo.***

In order to investigate the relative contributions of intrathymic infection versus infection of activated cells in the generation of circulating HIV infected CD8 lymphocytes, HIV DNA was quantified in subsets of CD8 lymphocytes. CD8 lymphocytes expressing CD4 ($CD8^{bright}CD4^{dim}$) were found to be infected in 7 out of 9 subjects (78%) with relatively high levels of infection (median 865 DNA copies/million cells, range undetectable – 3951; Table 4-4A, Figure 4-3), approaching those in the CD4 lymphocytes of the same 9 subjects (Figure 4-6A).

These high HIV proviral loads in the $CD8^{bright}CD4^{dim}$ lymphocyte populations were all greater than ten times that attributable to CD4 lymphocytes (Table 4-4A, Figure 4-4) and were largely retained when recalculated using the 'worst case scenario' method (Table 4-4A).

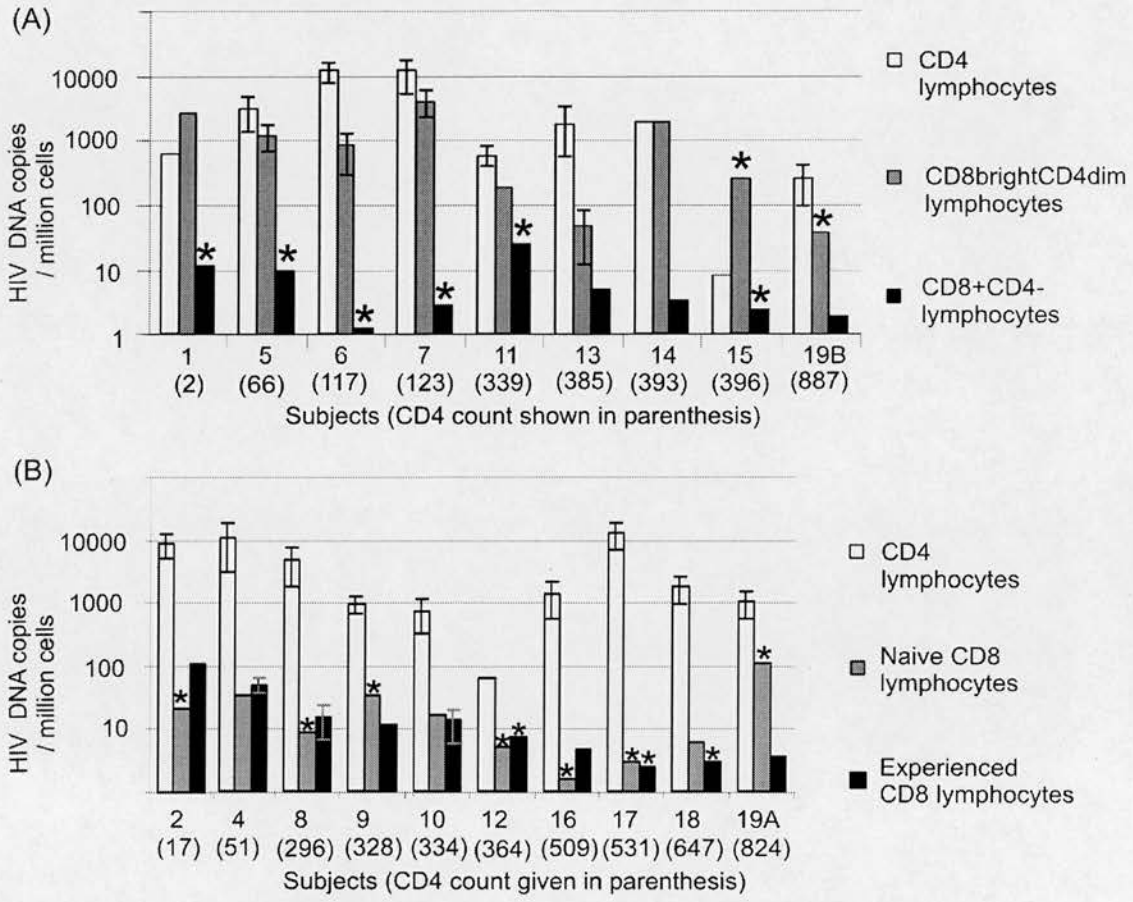


Figure 4-6. HIV DNA loads for each subject. Best estimate HIV DNA loads for CD4 lymphocytes and CD8 lymphocyte subsets for each subject (subjects ordered by ascending CD4 lymphocyte count) are given: (A) group 1 samples, (B) group 2 samples. Error bars indicate standard error (see methods), they are absent from samples where insufficient cells were available. * indicates samples where no virus was detected, the value given is half the lower limit of detection.

In contrast only three of nine subjects (33%) showed infection of $CD8^{+}CD4^{-}$ lymphocytes. The level of infection was low at 7, 5 and 2 HIV DNA copies/million cells, dropping to 4, 2 and 2 HIV DNA copies/million cells when contamination from $CD8^{bright}CD4^{dim}$ lymphocytes was taken into account. In all cases the proviral load attributable to $CD8^{+}CD4^{-}$ lymphocytes was less than ten times that attributable to CD4 contamination (Figure 4-4) and in two cases it became undetectable when

attributable proviral load was calculated using the 'worst case scenario' approach. The greater HIV DNA load in $CD8^{bright}CD4^{dim}$ lymphocytes as compared to $CD8\beta^{+}CD4^{-}$ lymphocytes was significant (Wilcoxon Signed Ranks Test: $p<0.01$ where undetectable values were assigned half the lower limit of detection, and $p<0.05$ where undetectable values were assigned zero).

Where CD8 lymphocyte subsets were divided on the basis of differentiation phenotype infection levels were generally quite low (range <3 – 110 HIV DNA copies/million cells, Table 4-4B. HIV DNA was detected in seven of ten antigen experienced samples compared to three out of ten antigen naïve samples, but there was no significant difference in HIV DNA load (Wilcoxon Signed Ranks Test, $p=0.7$, Figure 4-3C). The proviral load attributable to antigen experienced CD8 lymphocytes subsets was greater than ten times that attributable to contaminating CD4 lymphocytes in three of the seven subjects (Figure 4-4), and remained detectable in five subjects on use of the 'worst case scenario' method (Table 4-4B). In contrast, only one naïve CD8 lymphocyte sample had an attributable proviral load greater than ten times that expected from CD4 lymphocyte plus experienced CD8 lymphocyte contamination, and, in that sample, provirus became undetectable on calculation of attributable proviral load using the 'worst case scenario' method.

Further subdivision of the antigen experienced cells into memory or effector subsets was performed in four of the subjects with detectable HIV DNA. Of these, three demonstrated infection in the memory subset and one in the effector subset (Figure 4-3C), again with no significant difference in viral DNA load.

4.3.2.4 **HIV infection of CD8 lymphocytes in primary HIV infection.**

In the subject with PHI, the HIV DNA load in CD4 lymphocytes was 697 (SE 225) HIV DNA copies per million CD4 lymphocytes, a level in keeping with results from a previous series of 16 subjects with PHI (Karlsson *et al.*, 2001). The level of infection of $CD8^{bright}CD4^{dim}$ lymphocytes was similar to that of CD4 lymphocytes at 523 (SE 160) HIV DNA copies per million $CD8^{bright}CD4^{dim}$ lymphocytes. No HIV DNA was found in the CD8 lymphocytes not expressing CD4 (Figure 4-7).

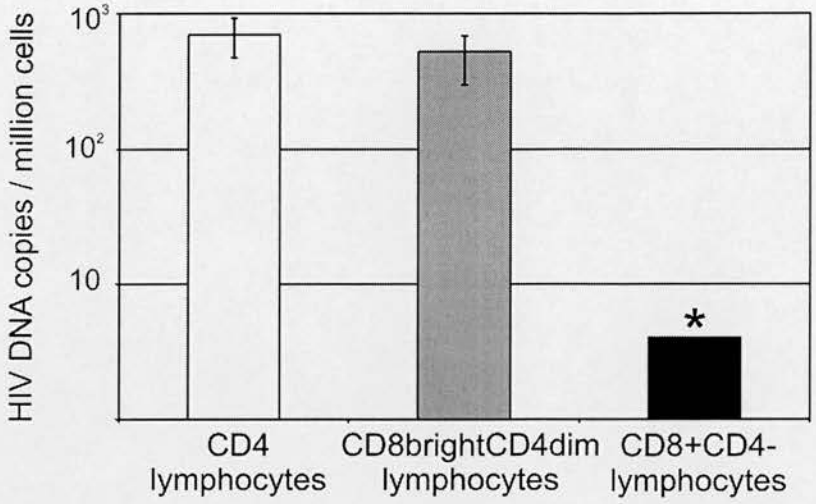


Figure 4-7. HIV DNA load in primary HIV infection. HIV DNA load attributable to CD4 lymphocytes, $CD8^{bright}CD4^{dim}$ lymphocytes and CD8+CD4- lymphocytes in a single subject with primary HIV infection. * indicates undetectable viral DNA, and half the lower limit of detection is given. Error bars show standard errors (see section 2.5.3).

4.4 Discussion

These results confirm that CD8 lymphocytes can be infected with HIV in subjects with both primary and chronic HIV infection, and that frequency of infection rises with disease progression. $CD8^{bright}CD4^{dim}$ lymphocytes have a high HIV proviral load similar to that found in CD4 lymphocytes, while $CD8^{+}CD4^{-}$ lymphocytes have either undetectable or very low levels of infection. HIV is more often detected in antigen experienced than antigen naïve CD8 lymphocytes. The level of confidence in these observations, and the interpretation of the distribution of HIV provirus between CD8 lymphocyte subsets are discussed here. Further consideration of the implications of these results for HIV immunopathology are given in Chapter 6:.

4.4.1 Level of confidence in estimated HIV proviral loads in CD8 lymphocyte populations.

Standard errors were calculated for the CD8 lymphocyte proviral loads (Table 4-), but these simply refer to the error intrinsic to the calculation of target sequence copy number from limiting dilution data, which is only the last step in the proviral load estimation protocol. To assess the degree of confidence in the results presented two

stages of the protocol must be considered: a) measurement of the purity of the CD8 lymphocyte populations, and b) quantification of the HIV LTR in the CD4 and CD8 lymphocyte populations. As described in detail below, errors at either of these stages could conceivably account for the provirus seen in $CD8^{+}CD4^{-}$ and naïve CD8 lymphocyte populations, but not those seen in $CD8^{bright}CD4^{dim}$ lymphocytes.

4.4.1.1 **Purity.**

The two step isolation protocol minimised CD4 lymphocyte contamination of the CD8 lymphocyte populations, and measurement of the levels of CD4 lymphocyte contamination allowed calculation of 'best estimate' proviral loads. The true level of contamination of CD8 lymphocyte populations with HIV infected cells could exceed that measured through the following 6 mechanisms: i) Some CD4 lymphocytes may fall within the CD4-ve quadrant on flow cytometry either due to downregulation of CD4 secondary to HIV infection, or due to bleaching of the anti-CD4 conjugated fluorochrome during the sorting procedure; ii) Some non-CD4 lymphocyte contaminants such as monocytes and γδ-T lymphocytes may carry HIV provirus; iii) There may be contamination with HIV infected CD8 lymphocytes of a different subtype; iv) Where gated data was used (ie for all $CD8^{bright}CD4^{dim}$ and $CD8^{+}CD4^{-}$ lymphocyte populations), some CD4 lymphocytes may lie outside the 'live cell' gate due to fragmentation during the sorting procedure; v) where the level of CD4 contamination could not be measured due to low cell numbers, the substitute level used may have been inaccurate; vi) The CD4 lymphocytes contaminating the CD8 lymphocyte populations may be different from the general CD4 lymphcyte population and therefore might have a higher HIV proviral load.

Before considering these potential influences in turn, it is worth noting that as the proviral load in $CD8^{bright}CD4^{dim}$ lymphocytes sometimes exceeded that of CD4 lymphocytes, it cannot be completely accounted for by any level of unselected CD4 lymphocyte contamination (ie. only mechanism (vi) can challenge the finding of HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes). In addition, the lack of a correlation between CD4 lymphocyte proviral load and CD8 lymphocyte proviral load argues

against CD4 lymphocyte contamination being the source of virus attributed to CD8 lymphocytes.

i) and ii). To determine how robust the data were to the first two challenges, 'worst case scenario' attributable proviral loads were calculated in which all non-CD8 lymphocytes were considered to carry as much HIV provirus as CD4 lymphocytes. Using this method HIV provirus attributable to CD8 was observed in 6/9 $CD8^{bright}CD4^{dim}$ populations, 0/9 CD8+CD4- populations, 5/10 experienced CD8 populations and 2/10 naïve CD8 populations (Table 4-). To assess the contribution of γδ-T-lymphocytes and monocytes to the observed HIV provirus, proviral loads were assessed in these cells in a subset of samples. HIV provirus was detected in γδ-T-lymphocytes in 1/10 subjects and in monocytes in 2/3 subjects, in all cases the proviral load was less than 50 copies per million cells, considerably less than observed in CD4 lymphocytes.

iii) Proviral load attributable to contaminating $CD8^{bright}CD4^{dim}$ lymphocytes did not completely account for the provirus observed in $CD8^{+}CD4^{-}$ lymphocyte populations, but further decreased the already low proviral loads attributable to $CD8^{+}CD4^{-}$ lymphocytes. Similarly, proviral load attributable to contaminating experienced CD8 lymphocytes did not completely account for the provirus observed in naive lymphocyte populations, though it did contribute sufficient to ensure that in all samples, proviral load attributable to naïve CD8 lymphocytes was less than ten times that attributed to contaminants.

iv) The use of gated purity data for $CD8^{bright}CD4^{dim}$ and $CD8^{+}CD4^{-}$ lymphocyte populations was necessary due to the high frequency of background events mimicking CD4 lymphocyte contamination (Figure 4-1). While this does allow the potential for contaminating cell fragments to be excluded from the purity measurement, gated data has been used elsewhere (Brenchley *et al.*, 2004a) and is likely to provide the most accurate measurement. Reassurance that these events were not contributing HIV DNA was provided by the lack of detectable provirus in $CD8^{+}CD4^{-}$ samples with high ungated levels CD4+CD8- contamination. ie. In samples 6 and 7 (which had high ungated levels CD4+CD8- contamination, Table 4-), if all CD4+CD8- events lying outside the live cell gate were true CD4

lymphocyte contaminants, then HIV proviral loads of 12 and 155 would have been detected. When tested, these populations revealed undetectable proviral loads with the lower limit of detection at 2 and 41 respectively.

v) CD4 lymphocyte contamination was not measured in 5/9 samples of $CD8^{bright}CD4^{dim}$ lymphocytes, 2/10 naïve and 3/10 experienced CD8 lymphocytes. Of these 4/5 $CD8^{bright}CD4^{dim}$ lymphocyte populations, neither of the naïve samples and 2 of the 3 experienced samples had HIV provirus attributable to CD8 lymphocytes. The substitute level of contamination used to estimate proviral loads in the two experienced samples were 0.2% for sample 9 (as the 75^{th} percentile of the contamination seen in all cell subsets) and 0.41% for sample 10 (as a level equivalent to that of the naïve population was assumed). Contamination levels of 0.9% and 1.8% respectively would have been required to negate attributable CD8 lymphocyte provirus, compared to a median level of 0.03% and a maximum level of 0.79% observed for this cell subset (Table 4-3B).

The substitute level used for estimation of attributable proviral load in the $CD8^{bright}CD4^{dim}$ lymphocytes was taken to be equivalent to that of the CD8+CD4- lymphocytes from the same subjects. While this was considered to be the best estimate, it can be seen that on average, the level of contamination of $CD8^{bright}CD4^{dim}$ lymphocytes exceeded that of CD8+CD4- lymphocytes (Table 4-3A). Recalculation of the attributable proviral load for these four samples using the worst observed level of contamination (ie 2.44%) negates the observation of provirus attributable to $CD8^{bright}CD4^{dim}$ lymphocytes in one case (sample 13), but in all other samples the provirus observed in the $CD8^{bright}CD4^{dim}$ lymphocytes remains greater than ten times that attributable to CD4 lymphocyte contamination (Figure 4-4).

vi) Selective contamination of CD8 populations with CD4 lymphocytes carrying a higher HIV proviral load than the general CD4 lymphocyte population could have a marked effect on the level of provirus attributable to CD4 lymphocyte contamination. Such selective contamination could conceivably occur through

contamination with dead or activated CD4 lymphocytes. Selection of dead cells was avoided by staining with a dead cell marker and sorting only live cells. Selective contamination with activated CD4 lymphocytes can be envisaged due to the greater adhesive properties of activated cells which could make them more susceptible either to non-specific staining or to formation of doublets with CD8 lymphocytes. Arguments against this possibility include the results of the mixing experiments (section 3.1.2.2), which show no doublet generation, and the use of isotype controls to assess levels of non-specific binding.

4.4.1.2 **Quantification of HIV proviral load in lymphocyte populations.**

The development and monitoring of the real-time PCR for quantification of HIV LTR is covered in section 3.3.1, demonstrating accuracy to within 2.0 times the true value in serial dilutions of standard samples, and good correlation between limiting dilution and real-time results (Figure 3-14). The spectrophotometer used to measue DNA concentration was found to consistently read to an accuracy of within 0.8 to 1.2 times the true value on a standard sample. Some of the DNA extracted from samples had 260:280 ratios suggesting a degree of protein contamination, which could have lead to an overestimation of the amount of DNA in the sample. This tended to occur more for samples with very few cells, which were generally the CD8 lymphocyte subsets, and would lead to an underestimation of the proviral load within the sample. Overall it is therefore reasonable to assume that where the provirus measured in the CD8 lymphocyte population is more than 10 times that attributable to CD4 lymphocyte contamination, inaccuracies in the measurement of proviral load are unlikely to account for the CD8 lymphocyte provirus. Thus, this mechanism is unlikely to account for the provirus found in $CD8^{bright}CD4^{dim}$ or experienced CD8 lymphocytes (Figure 4-4), though it could conceivably account for most of the virus attributed to CD8+CD4- and naïve subsets.

4.4.1.3 **Unintegrated HIV DNA**

As described in the introduction (section 1.3.3), following reverse transcription HIV DNA can either integrate into the host cell genome or form circular DNA structures.

These DNA rings can be detected by PCR methods using primers binding wholly within the LTR sequence, and therefore can be detected by the limiting dilution assay used here. However, the outer anti-sense primer for the real-time PCR assay bind within the gag gene, and therefore would not amplify DNA from LTR circles. The good correlation between limiting dilution and real-time PCR estimates of proviral load (Figure 3-14) indicates that unintegrated DNA circles do not make up a significant proportion of the LTR detected. A similar finding was made previously by comparing results of limiting dilution proviral load estimates using primers within the LTR, versus primers spanning the LTR-gag junction(Imlach *et al.*, 2001).

4.4.2 Extent of CD8 lymphocyte infection.

In keeping with previous reports, the overall frequency of HIV infection of CD8 lymphocytes was very low (Livingstone *et al.*, 1996; McBreen *et al.*, 2001), and was lower than that reported for lung derived CD8 lymphocytes (Semenzato *et al.*, 1995; Semenzato *et al.*, 1998). The inverse correlation between CD8 lymphocyte proviral load and CD4 lymphocyte count suggests increasing infection of CD8 lymphocytes with disease progression. This could be caused by increased viraemia, increased lymphocyte activation (Eggena *et al.*, 2005), or the generation of variants more suited to infection of CD8 lymphocytes (Zerhouni *et al.*, 2004).

4.4.3 Route of CD8 lymphocyte infection.

CD4 dependent HIV infection of CD8 lymphocytes has been demonstrated during intrathymic development (De Rossi *et al.*, 1990; Brooks *et al.*, 2001) and following activation of mature lymphocytes (Flamand *et al.*, 1998; Kitchen *et al.*, 1998; Zhang *et al.*, 2001; Imlach *et al.*, 2001). Given that CD8 lymphocyte precursors would be expected to lose CD4 before leaving the thymus, our observation of very low or undetectable viral loads in $CD8\beta^{+}CD4^{-}$ lymphocytes implies that this route contributes little if anything to the circulating CD8 lymphocyte proviral load. Similarly, export of intrathymically infected cells is thought not to be the source of HIV infected naïve CD4 lymphocytes (Brenchley *et al.*, 2004a). In contrast, the higher proviral loads observed in the $CD8^{bright}CD4^{dim}$ population provides

convincing evidence of infection of this subset, suggesting that infection of CD8 lymphocytes occurs following antigen specific activation.

This distribution of HIV provirus is in keeping with previous results in which $CD8^{+}CD4^{+}$ cells isolated by immunomagnetic methods were found to contribute a higher proportion to the overall proviral load than their CD4 –ve counterparts (Imlach *et al.*, 2001). Similarly in a recent paper, Brenchley *et al.*, (2004a), observed 5 to100 fold more proviral copies in $CD8+CD4^{dull}$ lymphocytes than in CD8 lymphocytes lacking CD4 expression. The results presented are not consistent with previous reports of significant infection of blood derived CD8 lymphocyte populations isolated by removal of all CD4 expressing cells (McBreen *et al.*, 2001). Lung derived CD8 lymphocytes purified by removal of CD4 expressing cells (with CD4 lymphocyte contamination levels of 0.01%), have also demonstrated significant levels of infection (Semenzato *et al.*, 1995; Semenzato *et al.*, 1998). While these cells were isolated from a different tissue compartment, the results do suggest that CD8+CD4- lymphocytes can be infected, unlike the results presented here. The reason for this discrepancy is unclear, but it is possible that the need to fix the cells reduced the sensitivity of the protocol used here in comparison to those used by McBreen and Semenzato. This does not challenge the main finding that the frequency of infection is clearly much greater in the $CD8^{bright}CD4^{dim}$ than the $CD8^{bright}CD4^{-}$ population.

The distribution of provirus between CD8 lymphocytes at sequential stages of differentiation can also provide information regarding the likely route of infection. The use of phenotypic markers to define differentiation stages of CD8 lymphocytes has been a topic of much debate (Hamann *et al.*, 1999b), and is discussed in section 3.1.1. The relationship between differentiation markers and memory versus effector function remains unclear (Appay *et al.*, 2002a), and while the labels 'memory' and 'effector' are retained for the $CD27^{+}CD45RA^{-}$ and $CD27^{-}CD45RA^{+}$ subsets respectively, a degree of functional overlap is likely.

Given that the results provide good evidence for HIV provirus in $CD8^{bright}CD4^{dim}$ but not $CD8^{+}CD4^{-}$ lymphocytes, and the hypothesis that $CD8^{bright}CD4^{dim}$ lymphocytes are generated through antigen specific activation, one would expect HIV provirus to

be detectable in antigen experienced CD8 lymphocytes but not in the antigen naive subset. Interestingly, although provirus was more frequently detected in antigen experienced populations, three out of eleven subjects also demonstrated provirus in the naïve subset. While the evidence for infection of these naïve subsets was not particularly strong (being either less than ten times that expected from contaminants, or lost on calculation of 'worst case scenario' contamination, section4.4.1), it does warrant consideration. It is possible that the observed HIV infected naïve CD8 lymphocytes could be cells early in the priming process that have upregulated CD4 but have not yet lost their naïve markers, or could represent naïve CD8 lymphocytes undergoing regenerative proliferation. Alternatively they could be non-activated CD8 lymphocytes which have either been infected within the thymus or through a CD4 independent route. These issues are further investigated in chapter 5.

The lack of correlation between CD4 lymphocyte proviral load and CD8 lymphocyte proviral load suggests that, although we propose that HIV enters both cells via the CD4 receptor, different factors influence the frequency of this event in the two cell types. The inverse correlation between CD8 lymphocyte proviral load and CD4 lymphocyte count indicates that disease progression may be one factor that favours CD8 lymphocyte infection. Teasing out the relative importance of the many other host, viral and therapeutic factors that are likely to influence CD8 lymphocyte infection would be of major value in future studies.

Chapter 5: Generation and differentiation of $CD8^{bright}CD4^{dim}$ lymphocytes *in vivo*.

5.1 Introduction

Given that $CD8^{bright}CD4^{dim}$ lymphocytes are targets for HIV infection, a clear understanding of their natural history is important. Current knowledge is derived from analysis of $CD8^{bright}CD4^{dim}$ lymphocytes generated *in vitro* (Flamand *et al.*, 1998; Kitchen *et al.*, 1998; Laux *et al.*, 2000; Sullivan *et al.*, 2001; Kitchen *et al.*, 2002; Zloza *et al.*, 2003), from investigation of $CD8^{+}CD4^{+}$ lymphocytes circulating *in vivo* (Scott *et al.*, 1983; Blue *et al.*, 1985; Matsui *et al.*, 1989; Sala *et al.*, 1993; Ortolani *et al.*, 1993; Macchi *et al.*, 1993; Tonutti *et al.*, 1994; Prince *et al.*, 1994; Weiss *et al.*, 1998; Mizuki *et al.*, 1998; Bagot *et al.*, 1998; Nascimbeni *et al.*, 2004), and from observations of similar cells in animals including mice, chickens, swine and monkeys (Zuckermann & Husmann, 1996; Ober *et al.*, 1998; Nam *et al.*, 2000; Hernandez *et al.*, 2001; Nascimbeni *et al.*, 2004).

As described in section 1.5.3, a number of investigators have now shown that activation of CD8 lymphocytes *in vitro*, results in the upregulation of CD4 on between 20 and 80% of cells, with response mediated through T cell receptor (TCR) binding (Flamand *et al.*, 1998). Naïve CD8 lymphocytes were the most responsive (Kitchen *et al.*, 1998), and upregulated CD4 in response to mitogens such as staphylococcal enterotoxin B (SEB), to co-ligation with anti-CD3 and anti-CD28 monoclonal antibodies (but not anti-CD3 alone) and to co-culture with allogenic dendritic cells.

When compared to non-responding ($CD8^{+}CD4^{-}$) lymphocytes, a greater proportion of $CD8^{bright}CD4^{dim}$ lymphocytes expressed activation markers such as CD25, CD38 and CD95, and over 90% had the antigen experienced CD45RO+ phenotype (Flamand *et al.*, 1998; Sullivan *et al.*, 2001). The upregulation of CD4 appeared to be temporary reaching a peak around day six post stimulation, but although the proportion of CD8 lymphocytes expressing CD4 dropped after this peak, it had not

reached baseline by day 27, the longest published period of observation to date (Flamand *et al.*, 1998). It is not known whether the decrease in proportion of cells expressing CD4 over time was due to preferential death of cells expressing CD4 or due to downregulation of CD4 expression.

The presence of $CD8^{+}CD4^{+}$ lymphocytes in the human circulation *in vivo* was first noted in 1985 (Blue *et al.*, 1985). Subsequently expansions of $CD8^{+}CD4^{+}$ lymphocytes have been reported associated with viral infections (Ortolani *et al.*, 1993; Weiss *et al.*, 1998), with autoimmune disease (Scott *et al.*, 1983; Matsui *et al.*, 1989), with malignancy (Mizuki *et al.*, 1998; Bagot *et al.*, 1998)and in healthy subjects (Sala *et al.*, 1993; Tonutti *et al.*, 1994; Prince *et al.*, 1994). A number of these reports distinguished between $CD4^{bright}CD8^{dim}$ cells (which expressed a CD8 molecule composed of two α subunits and appeared to be of CD4 lymphocyte lineage) (Tonutti *et al.*, 1994; Suni *et al.*, 2001), and $CD8^{bright}CD4^{dim}$ cells (which expressed a CD8 molecule composed of an α and a β subunit and appeared to be of CD8 lymphocyte lineage) (Tonutti *et al.*, 1994). Ortolani *et al.*, (1993), noted that in a series of 51 subjects with expanded CD8+CD4+ populations, expansion of the $CD8^{bright}CD4^{dim}$ lymphocyte population occurred in 13 subjects all of whom had acute EBV or CMV infection, while expansion of $CD4^{bright}CD8^{dim}$ lymphocytes was more common and non-specific, occurring in healthy subjects and those with a range of pathologies. In keeping with the *in vitro* data, the $CD8^{bright}CD4^{dim}$ lymphocytes in the expanded populations had antigen experienced activated phenotypes, with over 90% expressing the markers CD45RO and CD38. These expanded populations were temporary, having disappeared within 2 months of recovery from the acute illness (Ortolani *et al.*, 1993). While both Blue and Ortlani observed a small population of CD8+CD4+ lymphocytes in healthy individuals they were not further characterised. More recently Nascimbeni *et al.*, (2004), assessed phenotype, antigen specificity and cytokine secretion profiles of CD8+CD4+ lymphocytes in healthy subjects and subjects with HCV infection. They confirmed that these cells are generally antigen experienced but did not find an increased activation profile. Their findings are considered in more detail in section 5.4

Together, the *in vitro* and *in vivo* evidence suggests that naïve, and, to a lesser, extent experienced CD8 lymphocytes upregulate CD4 after antigen specific, mitogen or alloantigen induced activation to generate a temporary $CD8^{bright}CD4^{dim}$ population. The relationship between this temporary population and the $CD8^{bright}CD4^{dim}$ lymphocytes circulating in healthy individuals and individuals with chronic infection remains unclear.

In this chapter two aspects of the natural history of CD4 expression on CD8 lymphocytes are addressed. First the differentiation and activation phenotype of circulating $CD8^{bright}CD4^{dim}$ lymphocytes in healthy controls was determined. The specific question of interest was whether the differentiation and activation phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes circulating in healthy subjects supports the hypothesis of generation through temporary CD4 upregulation following antigen specific activation. Secondly the effect of HIV infection on prevalence and phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes was assessed

The differentiation phenotype of circulating $CD8^{bright}CD4^{dim}$ lymphocytes in healthy subjects was determined *ex vivo* using the markers CD45RA and CD27. Use of these markers is discussed in section 3.1.1. Activation was assessed in terms of cell size and expression of CD69 and CD38. Upon activation, small resting lymphocytes transform into larger blasts (Robbins, 1964), and an increase in cell size has been shown to be a sensitive marker of activation (Teague *et al.*, 1993). Blue *et al.*, (1985), demonstrated that the CD8+CD4+ phenotype was associated with an increase in cell size in mitogen stimulated T lymphocyte cultures. They also noted a marked increase in CD4 and CD8 coexpression on large as compared to small lymphocytes circulating in peripheral blood, but the proportion of circulating CD8+CD4+ lymphocytes that were large was not given, and no distinction was made between $CD8^{bright}CD4^{dim}$ and $CD4^{bright}CD8^{dim}$ lymphocytes.

The activation marker CD69 is a signalling molecule and is one of the earliest surface markers to appear on activated T lymphocytes (Lopez-Cabrera *et al.*, 1993). Expression begins approximately 8 hours after stimulation with alloantigen, reaches a peak within 16-24 hours and then, in the absence of ongoing stimulation, expression is rapidly lost (Craston *et al.*, 1997). CD69 was expressed on between 12

and 50% of $CD8^{bright}CD4^{dim}$ lymphocytes generated by stimulation with staphylococcal enterotoxin B (Flamand *et al.*, 1998; Sullivan *et al.*, 2001).

CD38 is a transmembrane glycoprotein with enzymatic, adhesion and signalling functions. It is upregulated on activated CD8 lymphocytes, is expressed on over 95% of $CD8^{bright}CD4^{dim}$ lymphocytes generated *in vitro* (Sullivan *et al.*, 2001), and also on the vast majority of $CD8^{bright}CD4^{dim}$ lymphocytes found in association with acute viral infections (Ortolani *et al.*, 1993). The expression of CD38 is much less transient than that of CD69, with approximately 2/3 of CD8 lymphocytes specific for a lytic EBV antigen remaining $CD38^{+}$ a year after acute infectious mononucleosis (Bharadwaj *et al.*, 2001). In HIV infection CD38 expression is a poor prognostic marker and is associated with residual viral turnover in subjects on combination antiviral therapy (Giorgi *et al.*, 1993; Liu *et al.*, 1997; Benito *et al.*, 2004).

There are a number of mechanisms by which HIV could influence the $CD8^{bright}CD4^{dim}$ lymphocyte population. First, given that these cells are generated on activation, chronic presence of HIV antigen and the persistent immune activation characteristic of HIV infection, could lead to an expanded $CD8^{bright}CD4^{dim}$ lymphocyte population. Alternatively, HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes could, if cytopathic, decrease the lifespan of these cells leading to an overall diminution of the population size. Finally, HIV infection of the $CD8^{bright}CD4^{dim}$ lymphocytes could have more subtle effects such as interference with differentiation, or downregulation of CD4 expression (Kitchen *et al.*, 2004). To assess whether there is any evidence supportive of these potential effects, the prevalence and phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes was determined in 16 HIV infected subjects and compared with healthy controls.

5.2 Materials and Methods

5.2.1 Subjects and samples.

Blood samples from eight healthy controls, were used to assess the activation and differentiation phenotype of circulating $CD8^{bright}CD4^{dim}$ lymphocytes. The effect of HIV infection on these parameters was assessed using samples from 17 HIV infected

subjects, 16 samples were used to assess differentiation and four to assess activation, (Table 5-1). In addition the prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes was assessed in one subject with primary HIV infection. All but one of the HIV infected subjects also donated blood for the experiments described in chapter 4, and details of the recruitment process are given in section 4.2. The healthy controls were recruited from the staff of the Laboratory of Clinical and Molecular Virology, Edinburgh, and were age matched with the HIV+ve subjects: mean age of controls = 36, mean age of HIV infected subjects used to assess differentiation phenotype = 40, (independent samples T test: $p = 0.3$), mean age of HIV infected subjects used to assess activation phenotype = 38 (independent samples T test: $p = 0.4$).

The prevalence, activation and differentiation phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes was obtained for healthy controls and eleven of the HIV infected subjects by flow cytometry of appropriately stained PBMCs (Table 5-1). For the remaining six HIV infected subjects insufficient cells were available to use a PBMC aliquot. In these subjects PBMCs immunomagnetically enriched for CD8 lymphocytes were stained with differentiation markers for cell sorting (section 2.2), prevalence and differentiation phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes was then assessed on the sorted CD8 lymphocytes. The method for isolation of PBMCs is given in section 2.1.2.

Subject	Group[a]	CD4 count	Viral load	Age	Recent or ongoing acute infection	Differentiation phenotype assessed in PBMCs or sorted CD8 lymphocytes.	Activation assessed in terms of cell size	Activation markers assessed
1	1	Not Applicable		24	none	PBMC	yes	yes
2	1			48	none	PBMC	yes	yes
3	1			27	conjunctivitis	PBMC	yes	yes
4	1			50	none	PBMC	yes	
5	1			34	none	PBMC	yes	
6	1			38	none	PBMC	yes	yes
7	1			33	gastroenteritis	PBMC	yes	
8	1			35	none	PBMC	yes	
1	2	2	75,000	47	Pulmonary tuberculosis	PBMC		
2	2	17	750,000	29	Pyrexia of unknown origin	PBMC		
4	2	51	23,100	43	Pneumonia	CD8 lymphocytes		
5	2	66	75,000	44	Clinical diagnosis of PML, JC virus not isolated	PBMC		
6	2	117	75,000	31	Oral candidiasis	PBMC		
7	2	123	330,000	32	None	CD8 lymphocytes		
9	2	328	<50	43	None	PBMC		
10	2	334	<50	34	None	PBMC	yes	
11	2	339	14,900	37	None	CD8 lymphocytes		
12	2	364	304	37	None	PBMC		
13	2	385	153	40	None	PBMC	yes	
14	2	393	403	40	Cellulitis	PBMC		
15	2	396	862	54	None	CD8 lymphocytes		
16	2	509	<50	38	Small bowel overgrowth	CD8 lymphocytes		
17	2	531	<50	38	None	CD8 lymphocytes		
18	2	647	13400	41	None	PBMC	yes	yes[b]
21	2	501	<50	36	None		yes	
20[c]	2	630	3,500,500	34	PHI			

Table 5-1. Subject Characteristics. (a) Group 1 are healthy controls, group 2 are HIV infected subjects. (b) PBMCs stained with monoclonal antibodies against CD69, CD71 and HLAII. (c) This subject had primary HIV infection (PHI), and only prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes was assessed.

5.2.2 Flow cytometry.

5.2.2.1 **Preparation and staining of cells.**

Flow cytometry was used to assess the prevalence, differentiation and activation phenotype of $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocyte. All cells were prepared and stained for flow cytometry by the standard method (section 2.3). In addition to the standard unstained and single stained samples, cells were stained as indicated (Table 5-2).

Phenotype to be assessed	**Name of stain combination**	**Fluorescent conjugated monoclonal antibodies**
Differentiation	APC isotype[a]	CD27-FITC; CD8β-PE; CD45RA-cychrome; isotype-APC
	Test sample	CD27-FITC; CD8β-PE; CD45RA-cychrome; CD4-APC
Activation	Isotype APC	CD27-FITC; CD8β-PE; CD45RA-cychrome; isotype-APC
	Isotype FITC	isotype-FITC; CD8β-PE; CD45RA-cychrome; CD4-APC
	Test CD69	CD69-FITC; CD8β-PE; CD45RA-cychrome; CD4-APC
	Test CD38	CD38-FITC; CD8β-PE; CD45RA-cychrome; CD4-APC
	Test CD45RO	CD45RO-FITC, CD8β-PE; CD45RA-cychrome; CD4-APC
Prevelence of $CD8^{bright}CD4^{dim}$ lymphocytes in subject with PHI[b]	Test sample	CD4-FITC, CD8beta-PE, CD3-cychrome.

Table 5-2. Antibodies used to stain cells for assessment of $CD8^{bright}CD4^{dim}$ lymphocytes. Of note the monoclonal antibody used to detect CD8 expression was specific for the β subunit which is only expressed on CD8 lymphocytes. (a) APC isotype was included for most PBMC samples. (b) Prevelence of $CD8^{bright}CD4^{dim}$ lymphocytes in all other subjects was assessed on the sample stained for differentiation phenotype.

5.2.2.2 **Defining $CD8^{bright}CD4^{dim}$ lymphocytes.**

The prevelance of $CD8^{bright}CD4^{dim}$ lymphocytes, defined as the number of $CD8^{bright}CD4^{dim}$ lymphocytes divided by the total number of $CD8^{bright}$ lymphocytes, was assessed in 8 healthy controls and16 HIV positive subjects. As insufficient cells were available to allow staining of a separate PBMC sample for assessment of CD4 expression in all HIV infected subjects, the prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes was assessed on sorted CD8 samples in six subjects. As these subsets had been sorted using a standard fsc/ssc lymphocyte gate, this gate was also used in all PBMC samples. Use of this lymphocyte gate excluded up to 65% (median 27%) of live $CD8^{bright}CD4^{dim}$ lymphocytes which lay outside the gate due to increased cell size. However, reanalysis of 8 control and 4 HIV infected samples using a wider gate did not alter the conclusions reached, and therefore the standard lymphocyte gate was retained.

As isotype APC samples were not available for all samples, $CD8^{bright}CD4^{dim}$ lymphocytes were gated by eye (Figure 5-1A). Where an APC isotype sample was available this was used to check the position of the $CD8^{bright}CD4^{dim}$ lymphocyte gate which typically contained no events. The $CD8^{bright}CD4^{dim}$ gate was drawn to exclude $CD4^{bright}CD8^{dim}$ events, which were prominent in a few subjects (Figure 5-1B). Due to spectral overlap between APC and CyChrome appropriate compensation lead to spreading of the CyChrome high events along the APC axis. This obscured up to 50% of the $CD8^{bright}CD4^{dim}$ events (Figure 5-1C).

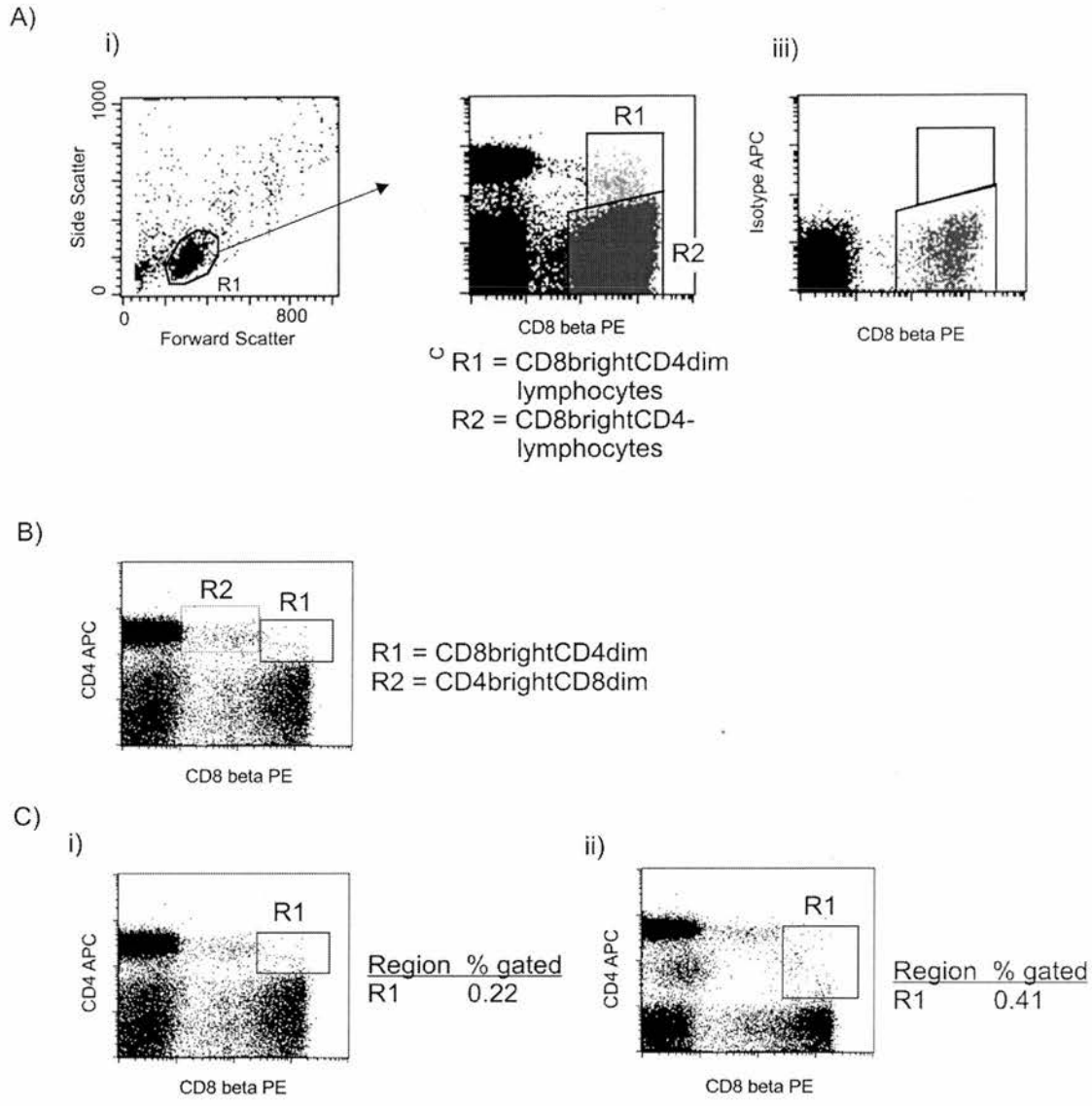


Figure 5-1. Defining $CD8^{bright}CD4^{-}$ and $CD8^{bright}CD4^{dim}$ lymphocytes. (A) The gating strategy used to define $CD8^{bright}CD4^{-}$ and $CD8^{bright}CD4^{dim}$ lymphocytes is shown. (i) A standard lymphocyte gate was used to ensure that data from PBMCs and sorted CD8 lymphocyte populations were comparable. (ii) $CD8^{bright}CD4^{-}$ and $CD8^{bright}CD4^{dim}$ lymphocytes were then gated by eye. (iii) Where an APC isotype stain was available this was used to check the position of the $CD8^{bright}CD4^{-}$ and $CD8^{bright}CD4^{dim}$ gates. (B) $CD8^{dim}CD4^{bright}$ lymphocytes were not included. (C) Spectral overlap between CyChrome and APC meant that up to 50% of the $CD8^{dim}CD4^{bright}$ lymphocytes were not assessed, dotplots of samples with (i), and without (ii) CD45RA cychrome staining are shown.

5.2.2.3 **Defining the differentiation phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes.**

The differentiation phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes was defined in terms of CD27 and CD45RA expression (Figure 5-2).

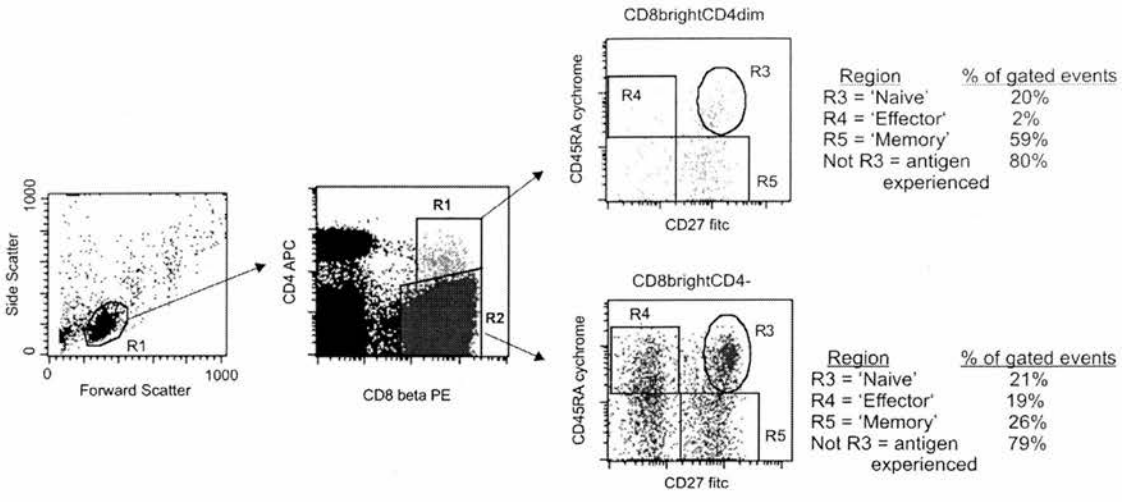


Figure 5-2. Defining differentiation phenotype of $CD8^{bright}CD4^{-}$ and $CD8^{bright}CD4^{dim}$ lymphocytes. A standard lymphocyte gate was used to ensure that data from PBMCs and sorted CD8 lymphocyte populations were comparable. $CD8^{bright}CD4^{-}$ and $CD8^{bright}CD4^{dim}$ lymphocytes were then gated by eye, and the proportion of these events expressing 'naïve' (CD45RA+CD27high), memory (CD45RA-CD27+) or 'effector' (CD45RA+CD27-) phenotypes was determined. Data from one healthy control subject.

5.2.2.4 **Defining activated lymphocytes in terms of cell size.**

The size of $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes was measured by flow cytometry in terms of fsc. To ensure that the population of CD8 lymphocytes with increased fsc did not contain increased numbers of dead cells (which could lead to non-specific staining and biased results) the fsc/ssc distribution of dead cells was determined by tubulin staining in one healthy and three HIV infected subjects. This demonstrated that in populations with increased ssc dead cells were common, while in those lying within a standard lymphocyte gate (termed 'small lymphocytes') or with increased fsc but only marginally increased ssc (termed 'large lymphocytes') dead cells were rare (Figure 5-3).

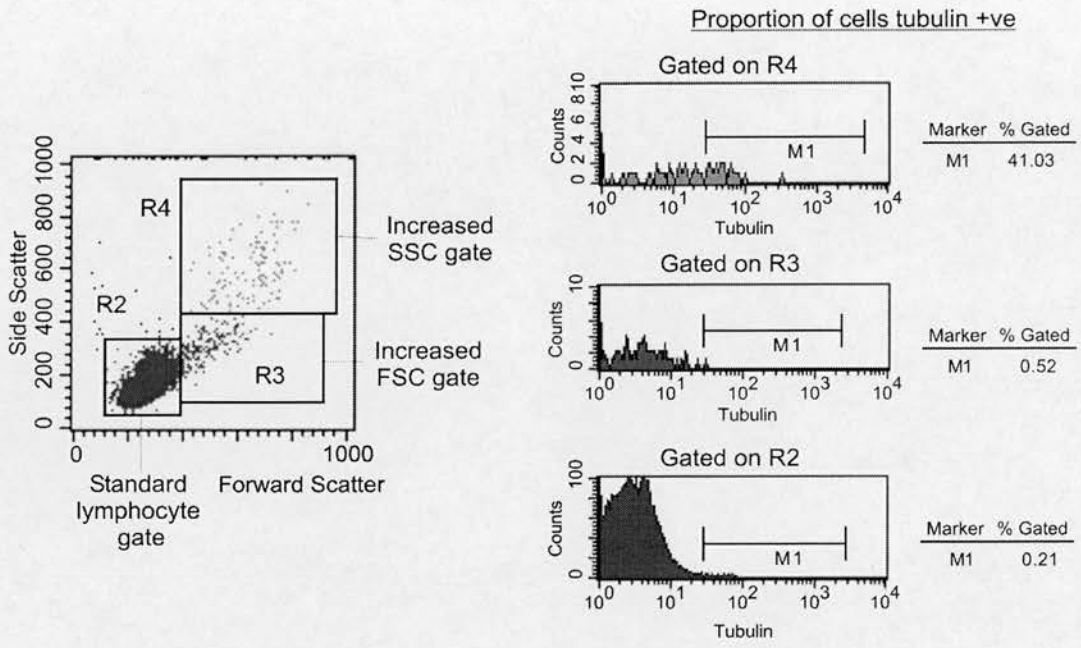


Figure 5-3. Distribution of dead cells in terms of fsc and ssc properties. Dead cells, defined by tubulin staining, were found to contribute a high proportion of cells with increased ssc, but contributed less the 1% of cells within a standard lymphocyte gate (small lymphocytes) or within an increased fsc gate (large lymphocytes).

To determine the relative proportions of 'small' and 'large' cells in the CD8 lymphocyte populations of interest, a two step process was used. First the position of the marker defining 'large lymphocytes' was determined using all CD8 lymphocytes with ssc characteristics of live cells. This marker position was then used to assess the relative number of 'large' and 'small' lymphocytes in $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocyte populations. The position of gates for a typical sample are shown in the results section (Figure 5-8).

To confirm that the 'large' CD8 lymphocytes defined as described above, were indeed more activated than their 'small' counterparts their expression of the activation markers CD69, CD38 and the differentiation marker CD45RO were compared. As expected, the proportion of cells expressing either the activation marker CD38 or the differentiation marker CD45RO, was consistantly higher in the CD8 lymphocytes with increased fsc than in small CD8 lymphocytes. This was true for both the $CD8^{bright}CD4^{dim}$ and the $CD8^{bright}CD4^{-}$ subsets. CD69 was expressed on a very small proportion of cells, and while numbers were too low to allow accurate

comparison of subsets, it was clear that the expression was not dramatically increased in large cells (Figure 5-4).

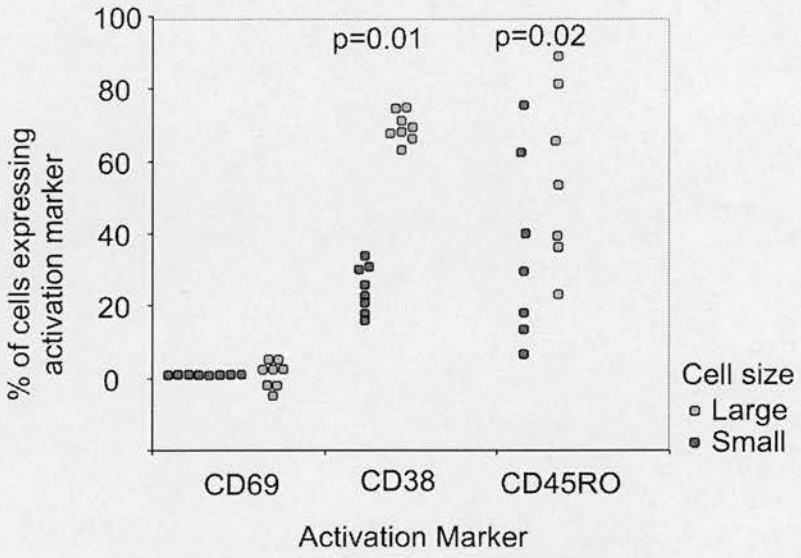


Figure 5-4. Proportion of 'large' and 'small' CD8 lymphocytes expressing activation markers. To confirm that the 'large' and 'small' populations do represent activated and quiescent populations respectively, the expression of activation markers on large and small $CD8^{bright}CD4^{dim}$ lymphocytes and $CD8^{bright}CD4^{-}$ were assessed in four subjects. Results for $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes are combined. Differences between groups was tested using Wilcoxon Sign Rank Test for paired cases.

5.2.2.5 Defining activated lymphocytes in terms of expression of activation markers.

Expression of the activation markers CD69 and CD38 and the differentiation marker CD45RO was determined for $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes using appropriately stained PBMCs from four healthy controls (Table 5-2). For this analysis lymphocytes with increased size were not excluded. Gating was performed as follows: (i) all events with a high ssc (largely dead cells) or a low fsc (cell fragments, and platelettes) were excluded; ii) $CD8^{bright}CD4^{dim}$ lymphocytes were gated by eye; iii) the marker defining the positive population for CD69, CD38 and CD45RO was set using the FITC isotype control, such that approximately 0.5% of events fell inside the marker bounds (Figure 5-10). To calculate the fraction of the

test cells that expressed the activation marker of interest, the fraction of FITC positive events in the isotype sample was subtracted from the proportion of FITC positive events in the test samples. In the case of CD69, where expression was very low, this sometimes resulted in a proportion less than zero.

5.2.3 Statistical analysis.

The distribution of differentiation and activation phenotypes within the lymphocyte subpopulations did not follow a normal distribution, and therefore results are described in terms of median values and ranges, and statistical analysis was performed by non-parametric tests: Mann-Whitney Test for unlinked cases or the Wilcoxon Signed Rank Test for paired cases.

5.3 Results

5.3.1 Prevalence of $CD8\beta^{bright}CD4^{dim}$ lymphocytes in healthy and HIV infected subjects.

The prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes ranged from 0.32 to 3.44% of total CD8 lymphocytes, and there was no significant difference between HIV infected and control subjects (median 0.7 and 1.1 for HIV infected and controls respectively, p= 0.34 Mann-Whitney test , Figure 5-5A). Within the HIV infected subjects no association was found between the prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes and either the CD4 lymphocyte count or the viral load, Figure 5-5B and C. The HIV infected subjects fell into two groups with the majority (13/16) having relatively few $CD8^{bright}CD4^{dim}$ lymphocytes (less than 1% of CD8 lymphocytes). One of the three subjects with higher $CD8^{bright}CD4^{dim}$ frequencies had acute pneumonia at the time of sampling, but the other two had no recent intercurrent infection. The CD4 counts and viral loads of these three subjects were unremarkable.

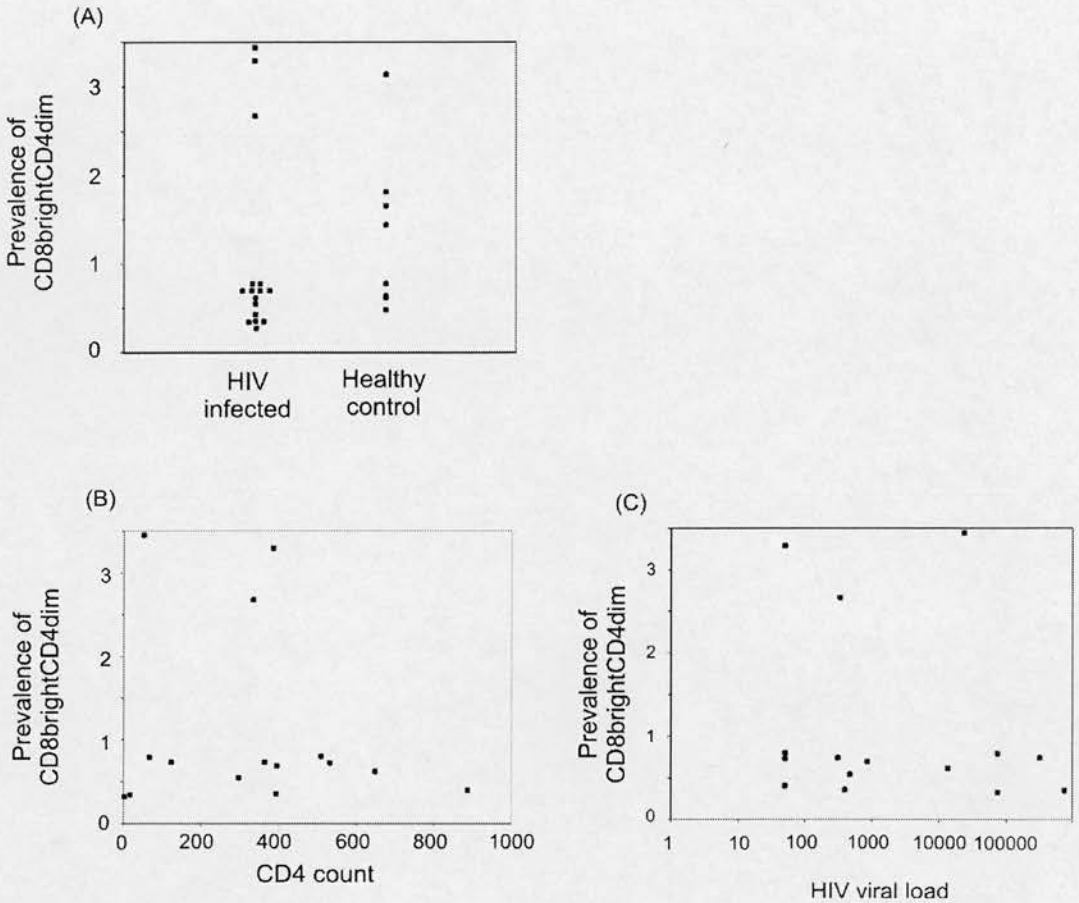


Figure 5-5. The proportion of CD8 lymphocytes expressing CD4 in HIV infected subjects and healthy controls. (A) The proportion of CD8 lymphocytes expressing CD4 was determined by flow cytometry in 16 HIV infected and 8 healthy controls. There is no significant difference between the two groups ($p = 0.034$, Mann-Whitney test). There was no significant association between the proportion of CD8 lymphocytes expressing CD4 and either CD4 lymphocyte count (B), or plasma viral load (C).

5.3.1.1 The prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes in PHI.

In the single subject with PHI, the prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes circulating in peripheral blood was 0.7% of CD8 lymphocytes, similar to that in subjects with chronic HIV infection.

5.3.2 *Differentiation phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes circulating in healthy and HIV infected subjects.*

To investigate the normal differentiation of $CD8^{bright}CD4^{dim}$ lymphocytes *in vivo* four colour flow cytometry was used to assess CD45RA and CD27 expression on $CD8^{bright}CD4^{dim}$ lymphocytes in PBMCs obtained from 8 healthy subjects. The effect of HIV infection on this subset was then determined using either PBMCs or sorted CD8 lymphocyte populations from 16 HIV infected subjects. No significant difference (Mann-Whitney Test) was found between the differentiation phenotypes displayed by $CD8\beta^{bright}CD4^{dim}$ lymphocytes assessed by these two different methods and thus the results were pooled and data is presented for all 16 subjects.

The majority of $CD8^{bright}CD4^{dim}$ lymphocytes in both healthy and HIV infected subjects displayed an antigen experienced phenotype (Figure 5-6A). This proportion was significantly greater than that in the $CD8^{bright}$CD4- lymphocytes in both the healthy and HIV infected subjects Wilcoxon Signed Ranks Test: $p=0.02$ and $p = 0.005$ respectively, Figure 5-6B).

Within the antigen experienced subset, the majority of $CD8^{bright}CD4^{dim}$ lymphocytes were CD45RA-CD27+ suggesting memory function while a small proportion were CD45RA+CD27- suggesting effector status. These figures represent a shift towards memory and away from effector phenotype when compared to antigen experienced $CD8^{bright}CD4^{-}$ lymphocytes, which was significant for HIV infected subjects (Wilcoxon Signed Ranks Test: $p= 0.003$ and $p = 0.001$ for the increase in memory and decrease in effector phenotypes respectively, Figure 5-6C).

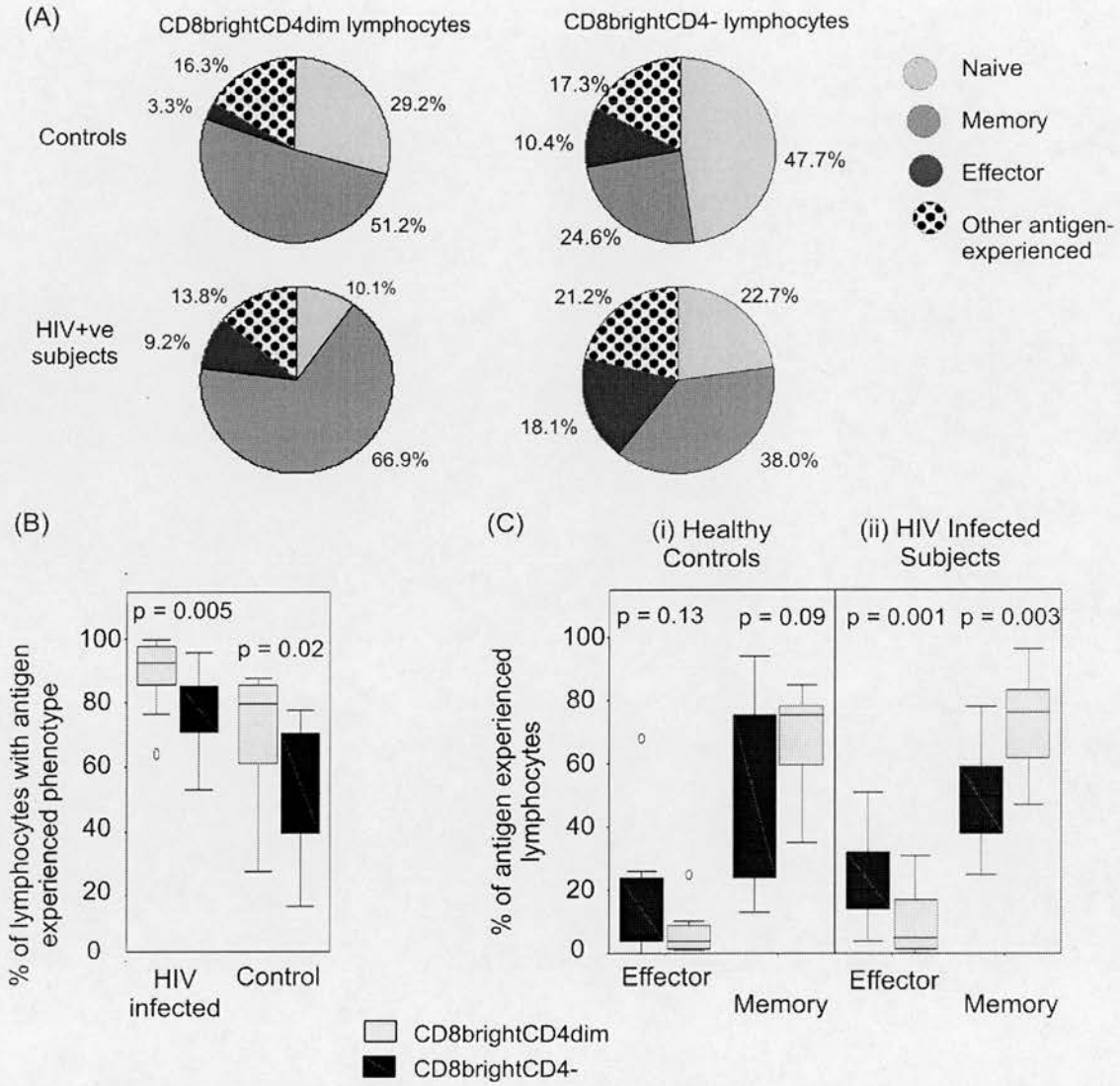


Figure 5-6. Differentiation of $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes in healthy and HIV infected subjects. (A) The proportion of $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes at various differentiation stages are shown as pie charts, the data used was the mean across all samples tested in control and HIV infected groups. (B) The range of results and difference between groups is shown as bar charts (B,C). The majority of $CD8^{bright}CD4^{dim}$ lymphocytes had an antigen experienced phenotype, and this represented a significant increase from that in $CD8^{bright}CD4^{-}$ lymphocytes in both HIV infected and healthy subjects (B). A greater proportion of $CD8^{bright}CD4^{dim}$ than $CD8^{bright}CD4^{-}$ antigen experienced lymphocytes had a memory phenotype, and less had an effector phenotype. This is true for both healthy controls (Ci) and HIV infected subjects (Cii), and was statistically significant for HIV infected subjects. p values are calculated using Wilcoxon Signed Rank test.

Direct comparison of the differentiation phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes in HIV infected versus healthy controls demonstrated a significant increase in the

proportion of cells with an antigen experienced phenotype (p = 0.005, Mann-Witney Test). However, this difference was a reflection of more general changes in the CD8 lymphocyte population of the HIV infected subjects (HIV is known to generate expanded antigen experienced lymphocyte populations), and when the prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes was expressed as a proportion of $CD8^{bright}CD4^{-}$ lymphocytes within each differentiation phenotype, there was no significant difference between HIV infected subjects and controls (Figure 5-7).

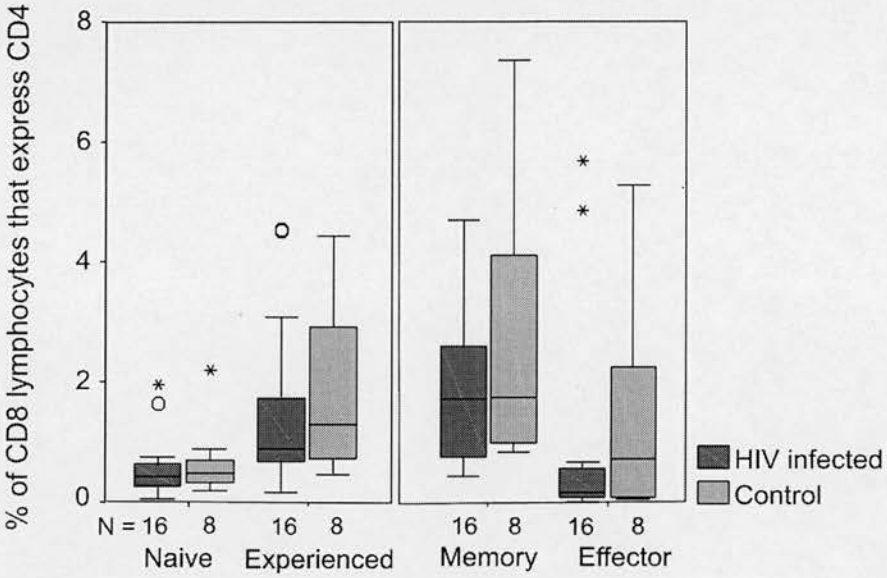


Figure 5-7. Proportion of CD8 lymphocytes expressing CD4, divided by differentiation phenotype. O represents outliers and * represents extreme outliers. Outliers are defined as values that lie more than 1.5 interquartile ranges beyond the quartiles. Extreme values lie more than 3 interquartile ranges beyond the quartiles.

5.3.3 The activation status of $CD8^{bright}CD4^{dim}$ lymphocytes and $CD8^{bright}CD4^{-}$ lymphocytes defined in terms of cell size.

$CD8^{bright}CD4^{dim}$ lymphocytes generated *in vitro* displayed an activated phenotype (Flamand *et al.*, 1998; Sullivan *et al.*, 2001). To determine whether the same was true of $CD8^{bright}CD4^{dim}$ lymphocytes in peripheral blood of healthy individuals, their degree of activation was assessed in terms of cell size in 8 subjects, and through expression of the activation markers CD69 and CD38 in four subjects (subjects 1,2,3 and 6). To assess whether HIV infection has any effect on the proportion of

$CD8^{bright}CD4^{dim}$ lymphocytes that are activated, cell size was also assessed in 4 HIV infected subjects.

$CD8^{bright}CD4^{dim}$ lymphocytes had a clear biphasic fsc distribution in both HIV infected subjects and controls (Figure 5-8). While a considerable proportion were large (median 27%, range 7 – 65%), suggestive of blast transformation, the majority were 'small' suggesting quiescence. A few $CD8^{bright}CD4^{-}$ lymphocytes were also large, but the proportion was significantly less (median 2%, range 1% – 4%) than found for the $CD8^{bright}CD4^{dim}$ lymphocytes (Wlcoxon signed ranks test: $p = 0.002$, Figure 5-9A).

The proportion of $CD8^{bright}CD4^{dim}$ lymphocytes with increased fsc in HIV infected subjects was significantly less than that in healthy controls, (medians 11.6% and 29.5% respectively, Mann-Whitney U Test: $p = 0.03$) . This was not the case for $CD8^{bright}CD4^{-}$ lymphocytes, (medians 1.1% and 2.0% for HIV infected and controls respectively; Mann-Whitney U Test: $p = 0.21$; Figure 5-9B).

The relative extent of activation of $CD8^{bright}CD4^{dim}$ as compared to $CD8^{bright}CD4^{-}$ lymphocytes was also assessed by simply comparing the mean fsc of the two populations, excluding cells with increased ssc. This allowed assessment of the relative cell size without recourse to defining 'small' and 'large' populations and was thus subject to less potential bias. Using this method the significant increase in size of $CD8^{bright}CD4^{dim}$ as compared to $CD8^{bright}CD4^{-}$ lymphocytes was confirmed (mean size 367 units and 307 units respectively, Wilcoxon Signed Ranks Test for paired samples $P = 0.002$). Similarly the significantly greater increase in size of $CD8^{bright}CD4^{dim}$ versus $CD8^{bright}CD4^{-}$ lymphocytes in control subjects compared to HIV infected subjects was confirmed (median proportional increase of fsc in $CD8^{bright}CD4^{dim}$ versus $CD8^{bright}CD4^{-}$ lymphocytes were 1.23 and 1.11 and for control and HIV infected subjects respectively, Mann-Whitney U Test: $p < 0.05$, Figure 5-9C).

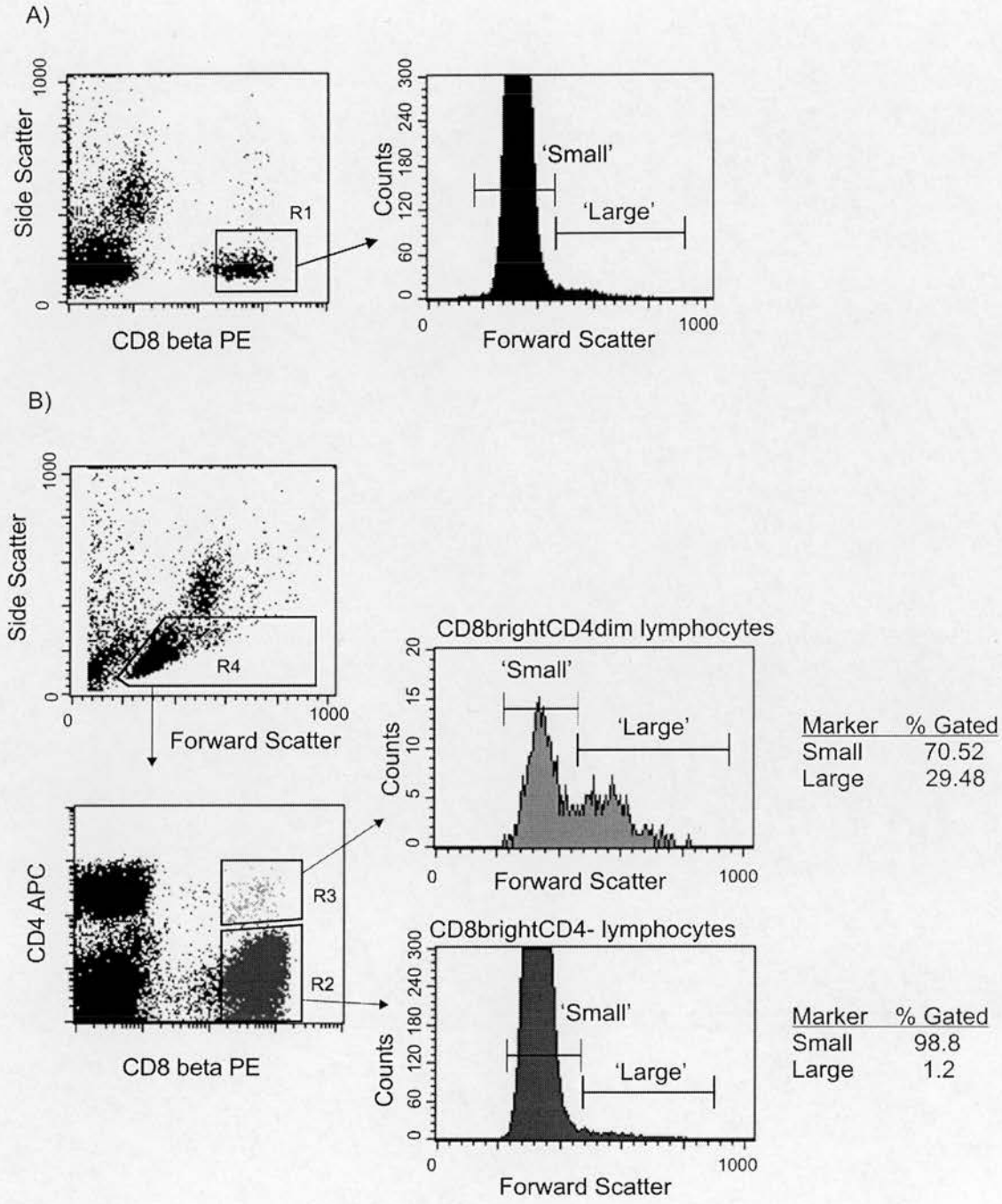


Figure 5-8. Proportion of $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocyte populations that have increased cell size. Results for a typical sample are shown. Cells from each subject were initially gated on $CD8^{bright}$ and ssc low, and a histogram of fsc obtained. This demonstrated a biphasic distribution, allowing events to be divided by eye into those with a standard lymphocyte fsc 'small', and those with increased fsc 'large'(A). The proportion of $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes falling within the 'small' and 'large' markers was then determined, (B). $CD8^{bright}CD4^{dim}$ lymphocytes show a clear biphasic fsc distribution with the majority being 'small'.

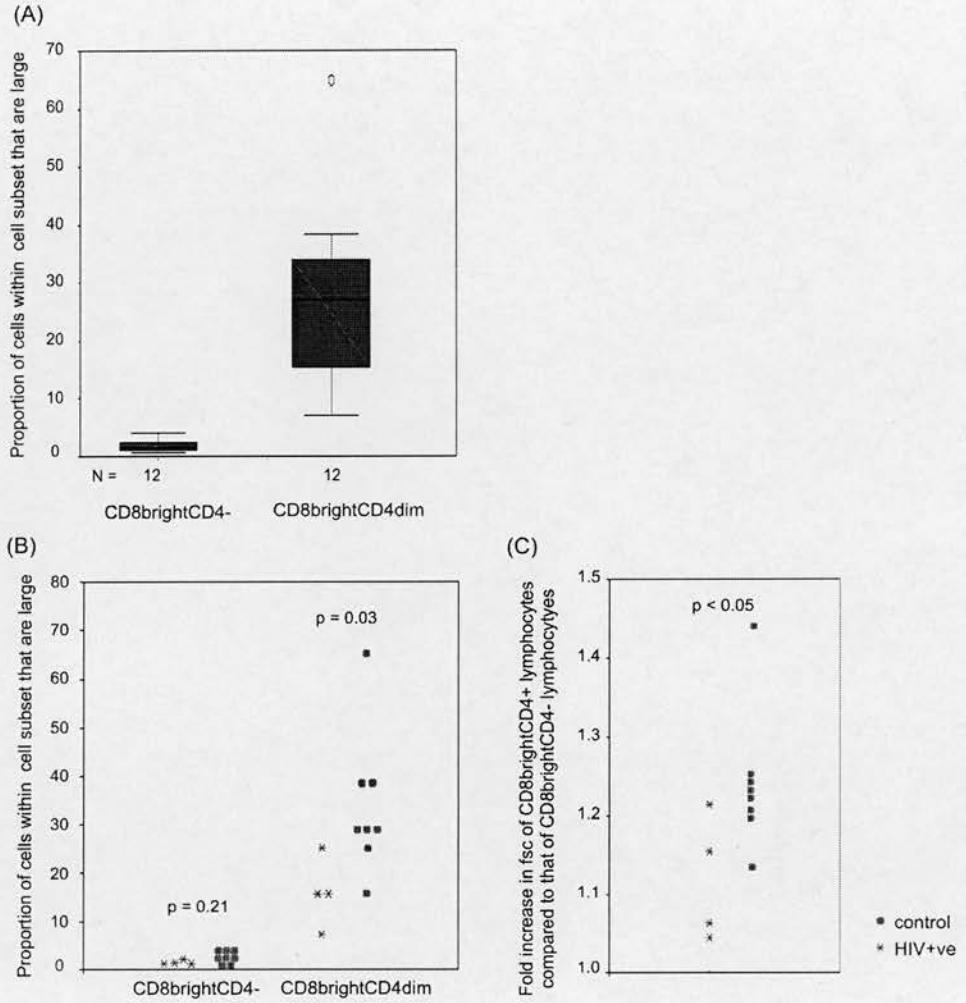


Figure 5-9. Cell size of $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes. The proportion of $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes that are 'large', suggesting blast transformation for HIV infected and control subjects (A). The same data divided into HIV infected and control subjects showing that the proportion of activated $CD8^{bright}CD4^{dim}$ lymphocytes in HIV infected subjects is significantly less than that of healthy controls (B). The difference between $CD8^{bright}CD4^{dim}$ lymphocytes of HIV infected subjects and controls is also seen when activation is measured as a relative increase in fsc (C).

5.3.4 Expression of activation markers.

The proportion of cells that expressed CD69, CD38 and CD45RO was assessed for $CD8^{bright}CD4^{dim}$ lymphocytes and $CD8^{bright}CD4^{-}$ lymphocytes.

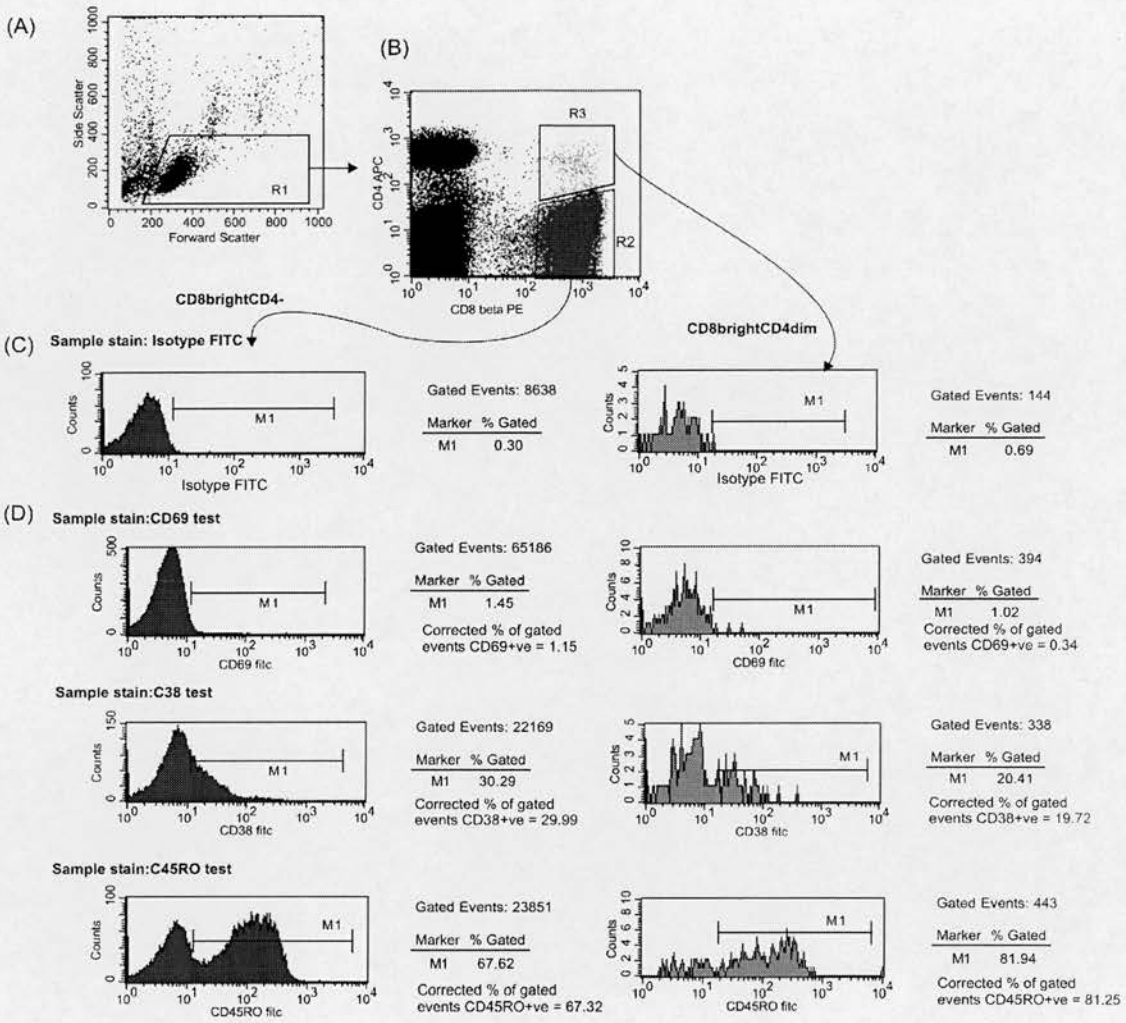


Figure 5-10. Positioning of gates for analysis of activation markers on $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes. Results for a typical sample are shown. Events with a high ssc or a low fsc were excluded (A). $CD8^{bright}CD4^{dim}$ lymphocytes were gated (B) and the marker defining the positive population for CD69, CD38 and CD45RO was set using the FITC isotype control (C). The proportion of FITC positive events in the control sample was then subtracted from the proportion of FITC positive events in the test samples to give a corrected proportion of the test population expressing the activation or differentiation marker (D).

Surprisingly, despite the increased proportion of large cells in the $CD8^{bright}CD4^{dim}$ populations compared to the $CD8^{bright}CD4^{-}$ populations, the proportion of cells expressing CD38 was no greater. This was in contrast to the expression of CD45RO which was consistently higher in $CD8^{bright}CD4^{dim}$ as compared to $CD8^{bright}CD4^{-}$ populations. Again the low level of staining with CD69 precluded accurate

comparison, but a marked increase in expression in $CD8^{bright}CD4^{dim}$ lymphocytes was excluded (Figure 5-11).

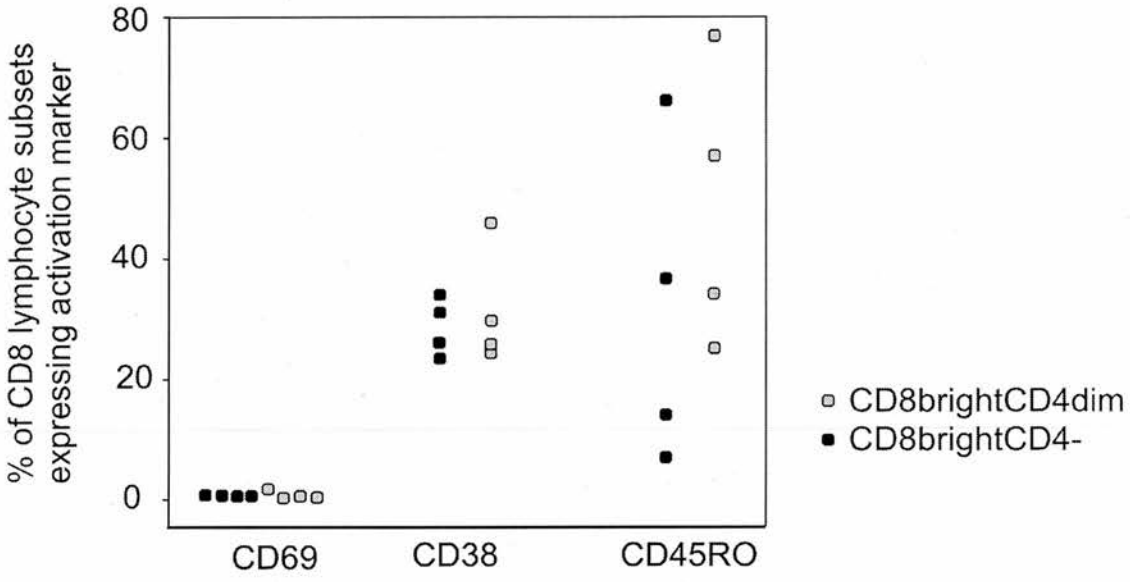


Figure 5-11. Expression of activation markers on $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-}$ lymphocytes. The proportion of $CD8^{bright}CD4^{dim}$ lymphocytes and $CD8^{bright}CD4^{-}$ lymphocytes expressing the activation markers CD69 and CD38 or the differentiation marker CD45RO was determined using appropriately stained PBMCs from four healthy controls.

Given the working hypothesis that $CD8^{bright}CD4^{dim}$ lymphocytes are generated by antigen specific activation, the observation of $CD8^{bright}CD4^{dim}$ lymphocytes with a naïve phenotype was unexpected. One explanation for this apparent contradiction is that the $CD8^{bright}CD4^{dim}$ lymphocytes displaying a naïve phenotype are cells that have recently encountered antigen (and thus upregulated CD4) but have not yet lost their naïve markers. If this were the case, one would expect naïve $CD8^{bright}CD4^{dim}$ lymphocytes to show evidence of recent activation. This was assessed in the eight healthy control subjects through measurement of the cell size. There were insufficient naïve $CD8^{bright}CD4^{dim}$ lymphocytes to perform these assessments in HIV infected subjects.

Assessment of forward scatter demonstrated that a considerable proportion (median 58%, range 15 – 88%) of naïve $CD8^{bright}CD4^{dim}$ lymphocytes were 'large',

suggesting blast transformation. This was not a general feature of naïve lymphocytes as very few (median 2%, range 0% -3%) naïve $CD8^{bright}CD4^{-}$ lymphocytes were 'large'. The increased size of naïve $CD8^{bright}CD4^{dim}$ lymphocytes as compared to naïve $CD8^{bright}CD4^{-}$ lymphocytes was confirmed by comparison of the mean fsc of the two CD8 lymphocyte subsets (median fold increase = 1.4).

Interestingly the proportion of naive $CD8^{bright}CD4^{dim}$ lymphocytes that were activated, as assessed by cell size, was significantly greater than that of antigen experienced $CD8^{bright}CD4^{dim}$ lymphocytes. Thus the proportion of experienced $CD8^{bright}CD4^{dim}$ lymphocytes that were large (median 18%, range 2 - 49%) was significantly less than that for naïve $CD8^{bright}CD4^{dim}$ lymphocytes, ($p = 0.001$ Paired Samples T Test, Figure 5-12), and this was confirmed by mean fsc measurements ($p = 0.001$ Paired Samples T Test). This difference in cell size was not a general feature of naïve and experienced cells as there was no significant difference in cell size of naïve and experienced $CD8^{bright}CD4^{-}$ lymphocytes ($p = 1$ Paired Samples T Test, Figure 5-12).

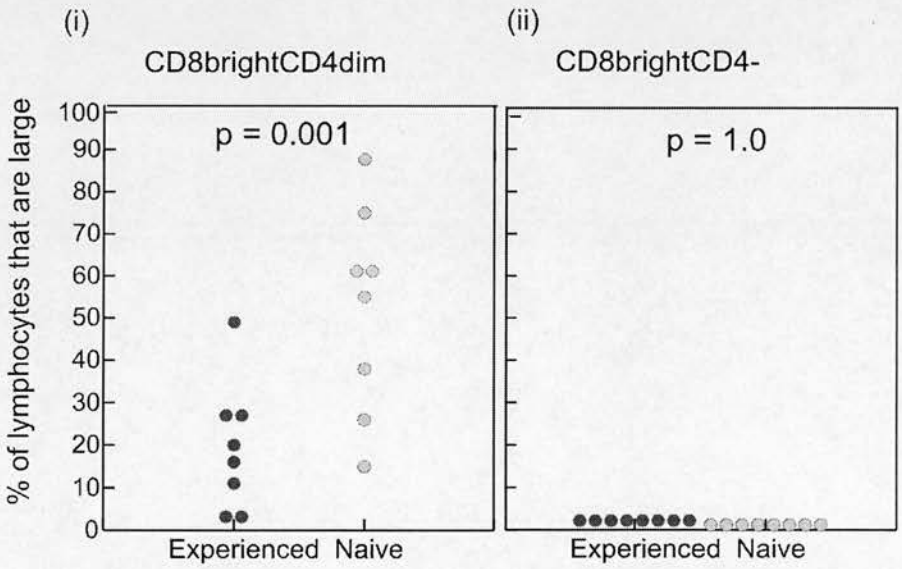


Figure 5-12. Size of naïve and experienced $CD8^{bright}CD4^{dim}$ lymphocytes. The activation status of naïve and experienced CD8 cell subsets was assessed in terms of cell size.

5.3.5 The level of CD4 expression on the $CD8^{bright}CD4^{dim}$ lymphocytes.

The level of CD4 expression on the $CD8^{bright}CD4^{dim}$ lymphocytes was just less than half (mean 0.41) that on true CD4 lymphocytes. The relative level of expression (level of expression on $CD8^{bright}CD4^{dim}$ lymphocytes / level on true CD4 lymphocytes) was less in HIV infected subjects than healthy controls (means 0.37 and 0.5 respectively $p = 0.03$, T test).

5.4 Discussion.

5.4.1 Prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes

The data presented show that $CD8^{bright}CD4^{dim}$ lymphocytes make up an average of 1.1% of CD8 lymphocytes in a small sample of healthy subjects. This result is in keeping with data from other series which have shown that $CD8^{bright}CD4^{dim}$ lymphocytes represent 0.15 – 0.9% of PBMCs, (Nascimbeni *et al.*, 2004). Other investigators who have determined the prevalence of all CD4+CD8+ lymphocytes (ie $CD8^{bright}CD4^{dim}$ and $CD4^{bright}CD8^{dim}$) have found higher levels, with average levels of approximately 1 – 3% of PBMCs (Blue *et al.*, 1985), with prevalence increasing with age (Laux *et al.*, 2000).

5.4.2 Differentiation phenotype of $CD8^{bright}CD4^{dim}$ lymphocytes

The results presented clearly show that the majority of circulating $CD8^{bright}CD4^{dim}$ lymphocytes were antigen experienced, supporting the hypothesis that $CD8^{bright}CD4^{dim}$ lymphocytes are generated through antigen specific stimulation. Within the antigen experienced subset, the memory phenotype predominated. The same observations were reported by Nascimbeni *et al.*, (2004), using CD45RO and CCR7 as markers of differentiation. They demonstrated that approximately 60% of $CD8^{bright}CD4^{dim}$ lymphocytes circulating in 10 healthy subjects displayed a memory phenotype, and also showed these cells tended to have an effector memory (tissue homing) rather than central memory (lymph node homing) expression pattern.

The relative lack of effector $CD8^{bright}CD4^{dim}$ lymphocytes suggests either that CD4 expression is lost on differentiation to effector status, or that expression of CD4 on

activation marks a branch point on the CD8 lymphocyte differentiation pathway, generating a CD8 lymphocyte subtype with a tendency to retain a memory phenotype. In a related finding, Zlosa *et al.*, (2003), show that $CD8^{bright}CD4^{dim}$ lymphocytes generated *in vitro* contain higher levels of IL-4 than their CD4-ve counterparts, with IL-4 secretion being a recognized function of memory but not effector CD8 lymohcytes (Geginat *et al.*, 2003). In swine, where mature CD4+CD8+ lymphocytes make up a significant proportion of circulating T lymphocytes, these cells appear to have a memory function, and specifically respond to viral recall antigens (Zuckermann & Husmann, 1996; Hernandez *et al.*, 2001).

Given the hypothesis that CD4 expression on CD8 lymphocytes occurs on antigen specific activation, it was a surprise to find that a considerable proportion (mean 16% over HIV infected and controls) of $CD8^{bright}CD4^{dim}$ lymphocytes displayed an antigen naïve phenotype. There are a number of possible explanations for this observation and the evidence for each is considered.

i) $CD45RA+CD27^{high}CD8^{bright}CD4^{dim}$ lymphocytes are recently antigen experienced but have either not yet lost their naïve markers. In support a significant fraction of the naïve $CD8^{bright}CD4^{dim}$ lymphocytes were 'large' suggesting recent activation (section 5.3.4). In addition it is known that early antigen experienced CD8 lymphocytes may retain CD45RA and CD27, that 10% of the cells designated 'naïve' by these markers are not 'true naïve' as assessed by further criteria, and that 12% of $CD8^{bright}CD4^{dim}$ lymphocytes generated *in vitro* retain CD45RA expression at least up to day 6 following stimulation (Sullivan *et al.*, 2001). The lack of CD69 expression could be taken as evidence against recent activation as CD69 is coexpressed with CD4 on mitogen stimulated CD8 lymphocytes *in vitro*. However it is known that this marker is often rapidly downregulated (within 48 hours of activation), and it is possible that in the *in vivo* environment CD69 expression is largely lost prior to the activated lymphocytes entering the circulation. CD38 expression can not be used to illuminate this issue as CD38 is constitutively expressed on naïve CD8 lymphocytes (Benito *et al.*, 1997).

ii) CD45RA+CD27^{high} CD8^{bright}CD4^{dim} lymphocytes are naïve CD8 lymphocytes undergoing regenerative proliferation. The naïve CD8 lymphocyte pool is maintained largely through low frequency regenerative proliferation. The expression of CD4 on proliferating naïve CD8 lymphocytes has not previously been described, but would explain the observed phenotype.

iii) CD45RA+CD27^{high} CD8^{bright}CD4^{dim} lymphocytes are antigen experienced but have reverted to a naïve phenotype. Reversion of antigen experienced T lymphocytes to phenotypically and functionally naïve cells has been demonstrated in the rat (Bell & Sparshott, 1990; Bunce & Bell, 1997) but there is no evidence that it occurs in humans.

iv) Some of the CD8^{bright}CD4^{dim} lymphocytes are not generated on activation but by some other mechanism. Mechanisms other than activation which could be responsible for generation of the circulating naïve CD8^{bright}CD4^{dim} lymphocytes include leakage of immature double positive thymocytes into the circulation. This was not investigated here, but previous studies have found no expression of thymic markers on circulating CD8+CD4+ lymphocytes (Ortolani *et al.*, 1993, Nascimbeni *et al.*, 2004).

v) Some of the CD8^{bright}CD4^{dim} lymphocytes observed by flow cytometry represent either coincidence artefact or non-specific staining. Although considerable attention was paid to ensuring that the observed CD8^{bright}CD4^{dim} lymphocytes were not artefactual (see section 3.1.2), it remains possible that artefact may be responsible for some of the CD8^{bright}CD4^{dim} events observed. Given that any non-specific staining would tend to generate events positive for all four markers used, artefactual 'naïve' events would predominate. Arguing against this explanation, in a single subject where a subset of PBMCs were stained for CD45RO, the number of CD8^{bright}CD4^{dim} lymphocytes within the CD45RO-CD45RA+ population (which would exclude indiscriminately stained events) was similar to that within the CD45RA+ population.

5.4.3 Activation status of $CD8^{bright}CD4^{dim}$ lymphocytes.

$CD8^{bright}CD4^{dim}$ lymphocyte populations produced *in vitro* have a fairly uniform activated phenotype. Thus by day 6 post stimulation a high proportion 12 – 60% (Flamand *et al.*, 1998; Sullivan *et al.*, 2001) expressed the early activation marker CD69, and over 90% expressed the later activation marker CD38 (Sullivan *et al.*, 2001). Assessment of $CD8^{bright}CD4^{dim}$ lymphocytes in subjects with a marked expansion in this cell type following acute viral infection demonstrated a similar phenotype to that seen *in vitro*, with over 90% expressing CD38 (Ortolani *et al.*, 1993).

This uniform activated phenotype was not found in the subjects assessed here. The cell size data indicated that the circulating $CD8^{bright}CD4^{dim}$ lymphocytes in healthy individuals was a mixed population, with a significant fraction (ranging from 6 – 65%) being 'large' suggesting activation, and the remainder being small in keeping with quiescence. The proportion of 'large' cells was significantly greater than that in $CD8^{bright}CD4^{-}$ lymphocytes, supporting the hypothesis that $CD8^{bright}CD4^{dim}$ lymphocytes *in vivo* are generated on activation. However, the presence of a considerable activated component was not confirmed by expression of activation markers. This could be due to the inability of the markers used to pick up the activated population. CD69 expression could be lost prior to migration of activated cells into the circulation, and as CD38 is constituitively expressed at a low level on naïve lymphocytes, any increased CD38 expression caused by activation could be masked by the decrease in naïve cells. Alternatively the activation marker data could correctly demonstrate the status of the $CD8^{bright}CD4^{dim}$ population and the cell size data could be at fault. Whichever dataset is the true representation, both agree that a large proportion of circulating $CD8^{bright}CD4^{dim}$ lymphocytes appear quiescent. Thus rather than being a wholly short-lived population, as suggested by the *in vitro* data, it appears that a subset of the $CD8^{bright}CD4^{dim}$ lymphocytes generated during antigen specific activation revert to a quiescent state and are thus likely to be long-lived. The existence of long-lived $CD8^{bright}CD4^{dim}$ lymphocytes is supported by their increasing prevalence with age, an increase which parallels the age related increment in memory lymphocytes (Laux *et al.*, 2000). Study of lymphocyte phenotype in a chimpanzee

during hepatitis C virus infection demonstrated that in addition to the temporary expansion in activated CD8+CD4+ lymphocytes during viraemia, there was a background population of relatively quiescent CD8+CD4+ lymphocytes.

Nascimbeni *et al.*, (2004), assessed expression of the activation markers DR, CD69, CD56 and CD38 and also found no significant difference in expresson between $CD8^{bright}CD4^{dim}$ and $CD8^{bright}CD4^{-ve}$ lymphocytes. This complete lack of increased expression of activation markers is hard to reconcile with the cell size data presented. It is possible that Nascimbeni *et al.* gated out activated cells by use of a tight lymphocyte gate during their analysis. An earlier report of the activation status of peripheral blood CD8 lymphocytes in healthy subjects did find a positive association between expression of CD69 and expression of CD4 (Imlach *et al.*, 2001). In this paper the proportion of CD8+CD4+ lymphocytes expressing each activation marker was not given, but review of some of the flow cytometry data indicates that the proportions were relatively low (Imlach,S. personal communication, 2004). Thus, this paper concurs that the degree of activation in the $CD8^{bright}CD4^{dim}$ lymphocytes circulating in healthy individuals is substantially less than that found in $CD8^{bright}CD4^{dim}$ lymphocytes generated *in vitro* or associated with acute infection. Imlach *et al.*, (2001), also found a correlation between expression of the later activation markers CD71 and HLAII, and expression of CD4 on CD8 lymphocytes. Using these markers to stain PBMCs from a single HIV infected subjects this association was not reproduced (data not shown).

A further difference between the $CD8^{bright}CD4^{dim}$ lymphocytes described here and those of acutely infected subjects relates to CD7 expression. Ortolani *et al.*, (1993), show that the $CD8^{bright}CD4^{dim}$ lymphocytes which expand following acute infection do not express CD7 (unlike their CD4-ve counterparts), while assessment of CD7 expression in $CD8^{bright}CD4^{dim}$ lymphocytes of a single HIV infected subject showed high levels of expression similar to those in $CD8^{bright}CD4^{-}$ lymphocytes. The relevance of this change in CD7 expression is not known.

5.4.4 *Effect of HIV infection on $CD8^{bright}CD4^{dim}$ lymphocytes.*

The relatively high HIV DNA load observed in $CD8^{bright}CD4^{dim}$ lymphocytes renders this population vulnerable to the cytopathic effects of the virus. However, in the small number of HIV+ve subjects and controls assessed there was no significant difference in the prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes, not for total CD8 lymphocytes or for any of the three differentiation stages assessed. This finding does not preclude a cytopathic effect of HIV on $CD8^{bright}CD4^{dim}$ lymphocytes, but indicates that if such an effect is present, it is counterbalanced by an increase in generation which would be a likely consequence of the high levels of immune activation seen in HIV infection.

In this regard, the finding that a smaller proportion of $CD8^{bright}CD4^{dim}$ lymphocytes were activated (as assessed by cell size) in HIV infected subjects compared to controls is of interest. In CD4 lymphocytes HIV is cytopathic to activated cells while quiescent cells may retain the provirus for long periods of time without ill effect (Chun *et al.*, 1995; Chun *et al.*, 1997) If a similar scenario operates for $CD8^{bright}CD4^{dim}$ lymphocytes, it could be responsible for the observed decrease in the ratio of activated to non-activated cells. While intriguing, this finding should be viewed with caution. It is based on a small sample size with HIV+ve subjects and healthy controls being matched only by age.

Given the hypothesis that $CD4^{bright}CD8^{dim}$ lymphocytes are generated on activation, and that activation markers such as CD38 have been shown to increase with HIV disease progression (Levacher *et al.*, 1992), one might expect there to be a correlation between the proportion of CD8 lymphocytes expressing CD4 and correlates of disease progression such as CD4 count or viral load. Such a correlation was not found in the small dataset presented here. This may reflect a true lack of association, but it is likely that any existent correlation could have been masked by the heterogeneity of the subjects in terms of treatment history and acute infective events. Of note Zloza *et al.*, (2003), did not report any correlation between the levels of $CD8^{bright}CD4^{dim}$ lymphocytes and markers of disease progression in their analysis of 150 HIV infected subjects.

The observed significant decrease in intensity of CD4 expression on CD8 lymphocytes of HIV infected as opposed to healthy controls is intriguing, though at this stage its significance is unclear. One could speculate that HIV infection of these cells is causing downregulation of the CD4 expression (as has been noted *in vitro;*(Kitchen *et al.*, 2004)), a suggestion that gains some support from a trend towards decreased CD4 intensity in subjects with increased CD8 lymphocyte viral load. However this trend does not reach significance (Spearman's rank correlation R = 0.54, p = 0.17), and further conclusions would need a much larger dataset.

The overall proportion of CD8 lymphocytes expressing CD4 found in HIV infected subjects here was lower than those reported in large retrospective surveys, but this is likely to be due to methodological differences. Zlosa *et al.,* (2003), showed that 0.4 – 3.4% of T cells are $CD8^{+}CD4^{+}$ in the majority of 150 HIV positive subjects, but this included $CD4^{bright}CD8^{dim}$ aswell as the $CD8^{bright}CD4^{dim}$ lymphocytes analysed here. They also reported a small number of subjects with higher frequencies, one reaching 16% of all T cells.

5.4.5 Limitations of the methods used.

While the four colour flow cytometry used here is an extremely powerful method to assess phenotype of lymphocyte subsets, the results presented do not reflect the entire population $CD8^{bright}CD4^{dim}$ lymphocytes for a number of reasons. First, in order to avoid contamination of the $CD8^{bright}CD4^{dim}$ lymphocyte population with non-specifically stained cells, CD8 bright lymphocytes with increased side scatter were excluded from all analyses. This excluded a mean of 56% of the total $CD8^{bright}CD4^{dim}$ events from the final assessment, with approximately half of the excluded events being tubulin positive (based on tubulin staining in a single subject, Figure 5-3). Given that $CD8^{bright}CD4^{dim}$ lymphocytes generated *in vitro* have been shown to have blast characteristics, which can increase side scatter aswell as fsc the exclusion of high ssc events is likely to have excluded a proportion of true $CD8^{bright}CD4^{dim}$ lymphocytes in addition to the artefactual events. In addition, cells with increased fsc (which represented on average 14% of $CD8^{bright}CD4^{dim}$ lymphocytes) were excluded from the analysis of the prevalence of $CD8^{bright}CD4^{dim}$

lymphocytes and their differentiation status. These exclusions, while necessary to ensure that the population examined did not contain artefactual events, may have skewed the results away from recently activated cells.

Secondly, as explained in section 5.2.2.2, compensation between spectral overlap of CyChrome and APC lead to spreading of the CyChrome high events along the APC detection axis. This made it more difficult to define a clear $CD8^{bright}CD4^{dim}$ population, and meant that a proportion of the lymphocytes with low levels of CD4 expression will have been missed, (Figure 5-1).

In addition to the exclusion of events of interest, the potential for artefactual events must be considered. As already discussed, the inclusion of high fsc events in the analysis of activation phenotype allows the possibility of inclusion of events representing doublets or coincidence error. While no evidence for this was found in mixing experiments (3.1.2), their possible confounding influence should be kept in mind.

Chapter 6: General Discussion

In this chapter the major findings of the experimental data are stated and a model of the dynamics of HIV infection of CD8 lymphocytes is proposed. The significance of HIV infection of CD8 lymphocytes is then considered in terms of immunodeficiency, viral reservoirs and viral replication. Finally potential directions for future research in this field are discussed.

6.1 Major findings of thesis.

In 16 subjects with chronic HIV infection, 0.3 – 3.4 % of blood derived CD8 lymphocytes expressed low levels of cell surface CD4, presenting a $CD8^{bright}CD4^{dim}$ phenotype. In general, these lymphocytes were infected with HIV at a level similar to that of CD4 lymphocytes. The frequency of infection increased with disease progression, and in subjects with advanced disease CD8 lymphocytes contained up to 25% of the total T lymphocyte proviral load. In the majority of subjects HIV infection of $CD8^{bright}CD4^{-}$ lymphocytes was not found, and where provirus was detected there were less than 20 copies per million cells. One subject with PHI also had HIV infected $CD8^{bright}CD4^{dim}$ lymphocytes, with levels of infection similar to those in chronic infection. As discussed in chapter 4, this pattern of infection of CD8 lymphocytes suggests infection following activation rather than export of intrathymically infected precursors.

In both healthy and HIV infected subjects, the majority of$CD8^{bright}CD4^{dim}$ lymphocytes were antigen experienced, and in contrast to the *in vitro* data, a significant proportion appeared quiescent. Chronic HIV infection had no demonstrable effect on their frequency or phenotype, and this cell type was not expanded in the subject with PHI.

6.2 Model of the dynamics of HIV infection of CD8 lymphocytes.

A proposed model of the dynamics of HIV infection of CD8 lymphocytes is given in Figure 6-1 and the accompanying table. The model is based on the data presented in this thesis, and published data, with the source of evidence given. As indicated, many of the events in the model occur in lymph nodes or at sites of inflammation, and therefore have to be inferred form data obtained from blood derived cells.

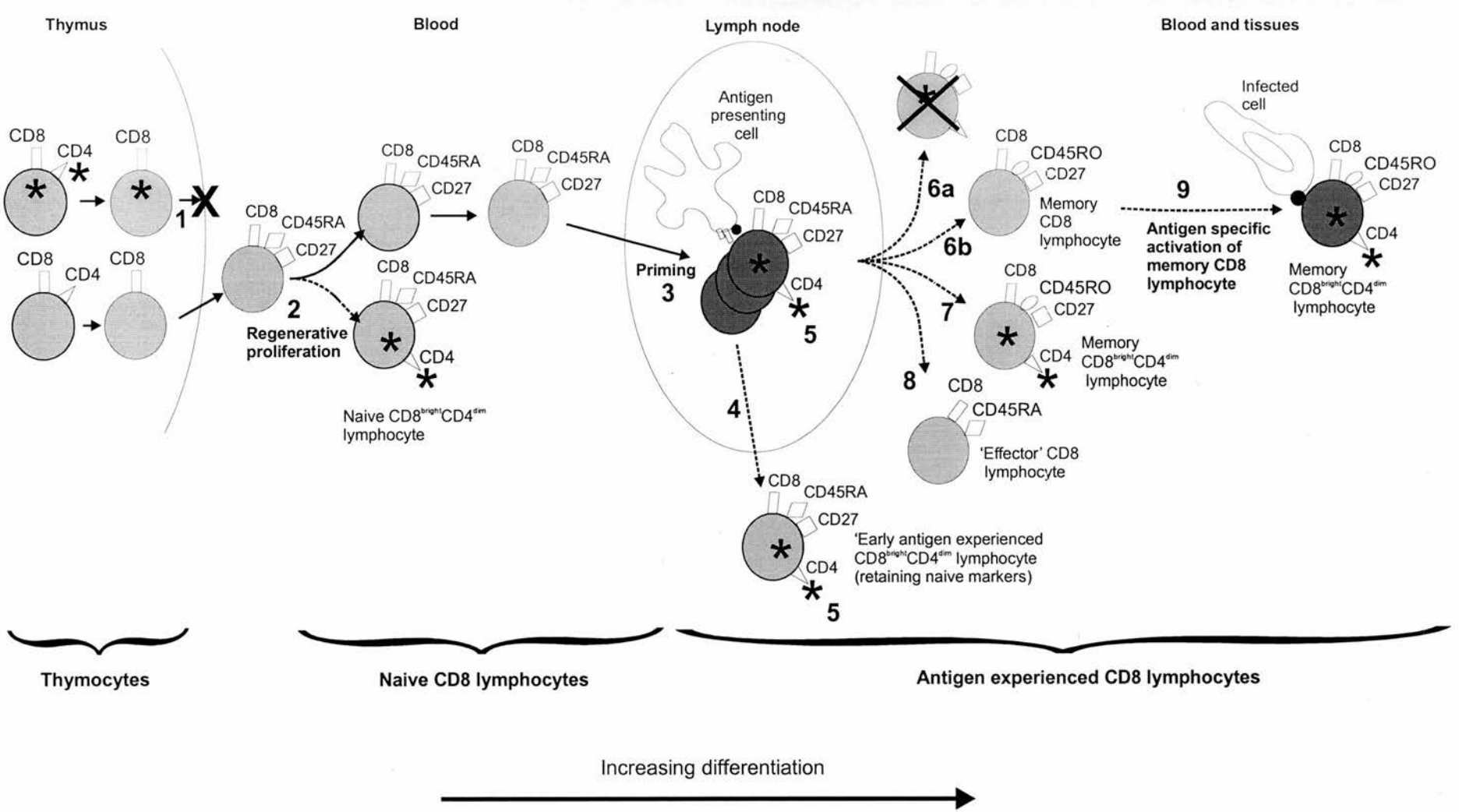


Fig 6.1. **A schematic diagram of the dynamics of CD4 expression on CD8 lymphocytes and their infection with HIV**. The maturation of CD8 lymphocytes from thymocyte to effector is depicted. The expression of CD4 and the differentiation markers CD27 and CD45 are indicated. Solid arrows depict events for which there is reasonable evidence, and dotted arrows depict possible events for which there is suggestive evidence. Activated cells are shown as darker grey circles. * indicates an HIV virion, when placed inside a cell it indicates a cell which may be infected, when placed next to a cell it indicates the cell is thought to be susceptible to infection. The numbers indicate events described in the table below.
express CD4 and are therefor susceptible to HIV infection. The numbers indicate events which are described in the table below.

Step	Description of event depicted in Figure 6-1	Evidence[a]
1	Intrathymically infected CD8 lymphocytes are not exported into the periphery.	*HIV infection of $CD8^{bright}CD4^{-}$ lymphocytes were not detected in the majority of subjects in this study (section 4.3.2.3).* Similar findings were reported by Brenchley et al (Brenchley *et al.*, 2004), but others found that $CD8^{+}CD4^{-}$ lymphocytes are HIV infected (Semenzato *et al.*, 1995; McBreen *et al.*, 2001; Imlach *et al.*, 2001).
2	CD4 may be upregulated on CD8 lymphocytes undergoing regenerative proliferation.	*$CD8^{bright}CD4^{dim}CD45RA^{+}CD27^{+}$ lymphocytes were detected in the circulation (section 5.4.2, see step 4 for an alternative interpretation of this observation).* A thymic source for circulating $CD8^{+}CD4^{+}$ lymphocytes has been excluded (Flamand *et al.*, 1998).
3	Antigen specific activation (priming) of naïve CD8 lymphocytes in lymphoid tissue may lead to upregulation of CD4 on up to 80% of cells	Upregulation of CD4 occurs on between 20 and 80% of CD8 lymphocytes following *in vitro* activation of CD8 lymphocytes (Flamand *et al.*, 1998; Sullivan *et al.*, 2001; Kitchen *et al.*, 2002)]. There is no *in vivo* data of the extent of CD4 expression on CD8 lymphocytes in lymph tissue.
4	CD4 upregulation occurs before loss of the naïve cell markers CD27 and CD45RA, and some of these cells enter the blood stream.	*$CD8^{bright}CD4^{dim}CD45RA^{+}CD27^{+}$ lymphocytes were detected in the circulation (section 5.4.2, see step 2 for an alternative interpretation of this observation).* A thymic source for circulating $CD8^{+}CD4^{+}$ lymphocytes has been excluded (Flamand *et al.*, 1998).
5	HIV infects $CD8^{bright}CD4^{dim}$ lymphocytes *in vivo*. This is likely to occur most efficiently in lymph tissue.	*HIV infected $CD8^{bright}CD4^{dim}$ lymphocytes were detected in the circulation (section 4.3.2.3).* Comparison with HIV infection of CD4 lymphocytes would suggest that infection is more efficient and occurs at a higher frequency in lymph tissue that in blood (Pantaleo *et al.*, 1993).
6 a&b	If 20-80% of CD8 lymphocytes upregulate CD4 on priming (as suggested by bulk stimulation experiments), then the vast majority of these cells must either die in preference to CD8 lymphocytes that have not upregulated CD4 (6a), or must downregulate CD4 on differentiation to long-lived antigen experienced cells (6b).	*Less than 2% of circulating antigen experienced CD8 lymphocytes express CD4 (section 5.3.1, fig 5.5).* If a significant proportion of $CD8^{bright}CD4^{dim}$ lymphocytes downregulate CD4 to become long-lived memory CD8 lymphocytes (as depicted in step 6b, one would expect to find HIV infected $CD8^{bright}CD4^{-}$ lymphocytes in the circulation. These were generally not detected suggesting either that step 6a predominates (programmed cell death), or that HIV infection of activated $CD8^{bright}CD4^{dim}$ cells is rapidly cytopathic. Arguing against step 6a, no increased apoptosis of *in vitro* generated $CD8^{bright}CD4^{dim}$ compared to $CD8^{bright}CD4^{-}$ lymphocytes was observed (Zlosa *et al.*, 2003).
7	Some of $CD8^{bright}CD4^{dim}$ lymphocytes differentiate into long-lived 'quiescent' memory lymphocytes retaining CD4 expression. These cells may be infected with HIV, and may remain susceptible to infection.	*$CD8^{bright}CD4^{dim}$ lymphocytes that were small and did not express the activation markers CD69 and CD38 were detected in the circulation (section 5.4.3).* There is no direct evidence that these quiescent $CD8^{bright}CD4^{dim}$ lymphocytes are infected, but data showing that the CD8 lymphocyte proviral load was stable over months of follow up, and the detection of ancestoral HIV variants in CD8 lymphocytes both suggest infection of a longlived population (McBreen *et al.*, 2001; Potter *et al.*, 2003).
8	$CD8^{bright}CD4^{dim}$ lymphocytes either do not differentiate to effector status, or differentiation to effector status is associated with greater downregulation of CD4 than is seen for memory cells.	*Compared to antigen experienced $CD8^{bright}CD4^{-}$ lymphocytes, significantly fewer $CD8^{bright}CD4^{dim}$ lymphocytes had an effector phenotype (section 5.3.2, fig 5.6).*
9	Antigen experienced CD8 lymphocytes may upregulate CD4 on re-encounter with cognate antigen presented with self MHC.	11% of CD45RA-ve (antigen experienced) CD8 lymphocytes upregulated CD4 on CD3/CD28 co-stimulation (Kitchen *et al.* 1988).

a) Italics indicate evidence presented in this thesis, the relevant section is quoted.

6.3 Significance of HIV infection of CD8 lymphocytes.

6.3.1 Immunodeficiency

HIV infection results in an as yet unexplained progressive decline in CD8 lymphocyte numbers and function. As reviewed in the introduction (sections 1.6.1 and 1.6.7.4), the decline in numbers largely affects naïve CD8 lymphocytes, and the functional defects include impaired proliferation and cytotoxicity. Various explanations for these deficits have been proposed, including lack of CD4 help, increased apoptosis and anergy, but none are backed by robust experimental evidence. The model presented above, where CD8 lymphocytes responding to antigen become infected with HIV, clearly has the potential to have a profound impact on the ability of CD8 lymphocytes to control pathogens.

Proponents of the 'tap and drain' or 'accelerated destruction' model argue that HIV induced death of activated CD4 lymphocytes leads to a decline in naïve CD4 lymphocyte numbers by an as yet undefined mechanism (see section 1.6.6.1) (Mohri *et al.*, 2001). While it is possible that infection of antigen experienced $CD8^{bright}CD4^{dim}$ lymphocytes could induce naïve CD8 lymphocyte decline in a similar manner, it is more likely that the decline in naïve cell numbers (both CD4 and CD8) is a consequence of HIV induced generalized immune activation (section 1.6.6.2). Alternatively, if CD4 is expressed on naïve lymphocytes undergoing regenerative proliferation, it is possible that HIV infection of these cells could directly impair naïve CD8 lymphocyte homeostasis. This hypothesis is supported by the finding, presented in section 5.4.1 and reported by Nascimbeni *et al.*, (2004), that significant numbers of circulating $CD8^{bright}CD4^{dim}$ lymphocytes have a naïve phenotype, and by the observation of HIV infection of naïve CD8 lymphocytes (presented in chapter 4). However upregulation of CD4 on regenerative proliferation has not been previously documented.

The extent of the impact depends to a large degree on four factors: the frequency of CD4 upregulation on CD8 lymphocytes, the proportion of $CD8^{bright}CD4^{dim}$

lymphocytes that become infected, the function of $CD8^{bright}CD4^{dim}$ lymphocytes, and the consequences of HIV infection at a cellular level.

6.3.1.1 Frequency and duration of CD4 upregulation on CD8 lymphocytes

In vitro bulk stimulation of CD8 lymphocytes with mitogen, CD3 and CD28 or allogenic cells results in CD4 upregulation on between 20 and 80% of cells (Flamand *et al.*, 1998; Kitchen *et al.*, 1998; Laux *et al.*, 2000; Sullivan *et al.*, 2001) but whether a similar proportion upregulate CD4 in response to antigen specific stimulation *in vivo* remains unknown. The stimulants used *in vitro* act through the TCR (Flamand *et al.*, 1998) and therefore mimic antigen specific priming, but the differences in receptor binding, the high concentrations of stimulant used, and the lack of additional costimulatory or inhibitory signals provided by the lymph node environment, mean that the *in vitro* studies can only provide an indirect indication of the extent of CD4 upregulation *in vivo*.

Animal studies have demonstrated that stimulation of lymphocytes *in vivo*, for example through vaccination, does lead to expansion of a $CD8^{+}CD4^{+}$ lymphocyte population (Periwal & Cebra, 1999). However, there is considerable interspecies variation in the extent of the expansion and the lineage of the $CD8^{+}CD4^{+}$ cells, for example $CD8^{+}CD4^{+}$ lymphocytes in mice and rats may be produced by premature release of thymocytes into the periphery (Bonomo *et al.*, 1994; Jimenez *et al.*, 2002), or in response to antigen specific activation (Periwal & Cebra, 1999), while in swine there is evidence that $CD8^{+}CD4^{+}$ lymphocytes are CD4 lymphocytes that have upregulated CD8 (Zuckermann & Husmann, 1996) (Hernandez *et al.*, 2001).

The observation of expanded $CD8^{bright}CD4^{dim}$ lymphocyte populations in subjects with acute EBV and CMV infections demonstrate that CD4 upregulation can occur on greater than 15% of circulating CD8 lymphocytes in *in vivo* (Ortolani *et al.*, 1993). However it is not known what proportion of subjects with acute EBV, CMV or other viral or bacterial infection have increased levels of $CD8^{bright}CD4^{dim}$ lymphocytes as no systematic study has been performed.

Levels of circulating $CD8^{bright}CD4^{dim}$ lymphocytes may under-represent total body frequencies, with greater frequencies expected in lymph nodes where naïve CD8

lymphocytes are primed. Again there is very little published data to support or refute this suggestion. In an SIV infected macaque with unusually high frequencies of $CD8^{bright}CD4^{dim}$ lymphocytes, the frequency in blood was greater than that in lymph node (Khatissian *et al.*, 2003). Conversely in a single HIV infected person, the proportion of CD8 lymphocytes expressing CD4 in lymph node was 4.6%, considerably more than is commonly found in blood (Hughes, G. 2005).

It is also reasonable to suppose that levels of $CD8^{bright}CD4^{dim}$ lymphocytes may be greater in the tissues than in blood, as tissues are the site of activation of primed CD8 lymphocytes, and $CD8^{bright}CD4^{dim}$ lymphocytes have increased expression of the tissue homing molecule CXCR3 (Nascimbeni *et al.*, 2004). Arguing against this, Semenzato found that $CD8^{+}CD4^{+}$ made up only 0.1 – 0.04% of bronchio-alveolar lavage cells in 35 HIV infected subjects with pulmonary symptoms (Semenzato *et al.*, 1995).

The duration of CD4 upregulation on CD8 lymphocytes would be expected to correlate with the likelihood of their becoming HIV infected. The lack of activation markers and small cell size of the majority of circulatng $CD8^{bright}CD4^{dim}$ lymphocytes (data presented in section 5.3.3), suggests that CD4 expression may be retained for a considerable period on cells that 'rest down' after antigen specific activation, a conclusion supported by the observed accumulation of $CD8^{bright}CD4^{dim}$ lymphocytes with age (Laux *et al.*, 2000). Whether these quiescent type cells remain susceptible to infection, or whether they enter phase $G_{(0)}$ of the cell cycle rendering them HIV resistant is unknown.

6.3.1.2 **Frequency of HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes.**

Data presented in chapter 4 demonstrated that the average level of infection of circulating $CD8^{bright}CD4^{dim}$ lymphocytes was 1197 proviral copies per 10^6 CD8 lymphocytes, or assuming a single proviral copies per cell (Simmonds *et al.*, 1990), a prevalence of infection of 0.1%. At first sight this appears a low infection prevalence, and one unlikely to have a profound impact on function of this CD8 lymphocyte subset. However, this 0.1% refers only to the proportion infected in the blood at the time the blood was drawn, it gives no indication of the proportion

infected in other tissues, and gives no indication of the incidence of infection, or the turnover of infected cells. Given that HIV infection is more efficient in activated cells which are prevalent in lymph nodes and inflamed tissue, it is likely that the proportion of $CD8^{bright}CD4^{dim}$ lymphocytes infected in these areas is greater than that in blood. This supposition is supported by reports of proviral loads in CD8 lymphocytes isolated from lung that were 2-6 fold higher than those in CD8 lymphocytes isolated from blood (Semenzato *et al.*, 1995; Semenzato *et al.*, 1998). Further support is provided by comparison with HIV and SIV infection of CD4 lymphocytes. Thus, in chronic infection the prevalence of HIV infected CD4 lymphocytes in lymph nodes is 3 – 10 fold that in blood (Pantaleo *et al.*, 1991), while in acute SIV infection up to 60% of CD4 lymphocytes residing in gut are infected compared to 30% in blood {Veazey, Tham, et al. 2000 19717 /id}. Infection incidence is much harder to ascertain, and is not known for any lymphocyte subset in HIV or SIV infection.

Of note, the proviral load in $CD8^{bright}CD4^{dim}$ lymphocytes was similar to that in CD4 lymphocytes, and given that direct infection is argued to be one of the causes of CD4 lymphocyte decline (Mohri *et al.*, 2001) the same case could be made for $CD8^{bright}CD4^{dim}$ lymphocytes.

6.3.1.3 **Function of $CD8^{bright}CD4^{dim}$ lymphocytes**

If $CD8^{bright}CD4^{dim}$ lymphocytes have specific functional abilities that do not overlap those of $CD8^{bright}CD4^{-}$ lymphocytes or CD4 lymphocytes, then even if they represent a small fraction of the total CD8 population, their loss could jeopardize immune integrity. Studies of the function of *in vitro* generated $CD8^{bright}CD4^{dim}$ lymphocytes have demonstrated antigen specific binding and a cytokine secretion profile dominated by IL-4, with minimal or absent secretion of IL-2, IL-10 and IFN-γ. 10-30% of $CD8^{bright}CD4^{dim}$ lymphocytes contained perforin (as did a similar proportion of $CD8^{bright}CD4^{-}$ lymphocytes) {Zloza, Sullivan, et al. 2003 23916 /id}). This profile is in contrast to that observed in *ex vivo* $CD8^{bright}CD4^{dim}$ lymphocytes where secretion of IFN-γ and TNF-α but not IL-4 or IL-10 was observed in response to a variety of recall and persistent antigens (Nascimbeni *et al.*, 2004). These discrepant findings are not altogether unsurprising given the different histories of the cell

populations tested, but do indicate that assigning function to this subset is likely to be a complex task. A specific function is supported by animal studies, in swine the double positive cells generated on activation function as memory cells with T helper function (Zuckermann & Husmann, 1996; Hernandez *et al.*, 2001), and in mice they have antigen specific cytotoxic ability (Periwal & Cebra, 1999).

In CD4 lymphocytes the CD4 molecule plays a vital part in activation, adhesion and migration (Center *et al.*, 2000; Konig & Zhou, 2004), and there is now evidence that it has similar functions in $CD8^{bright}CD4^{dim}$ lymphocytes. Using microarrays, Kitchen et al. demonstrated that CD4 ligation on $CD8^{bright}CD4^{dim}$ lymphocytes led to upregulation of genes encoding IFN-γ and Fas-L, while blocking of CD4-MHC-II interaction inhibited $CD8^{bright}CD4^{dim}$ lymphocyte cytotoxicity (Kitchen *et al.*, 2004). $CD8^{bright}CD4^{dim}$ lymphocytes were also shown to migrate along an IL-16 gradient by a CD4 dependent mechanism (Kitchen *et al.*, 2002). IL-16 is secreted by epithelial cells and eosinophils at sites of inflammation, and is known to induce migration of CD4 lymphocytes to these sites (Franz *et al.*, 1998). Interestingly, CD4 also directs the migration of both CD4 lymphocytes and $CD8^{bright}CD4^{dim}$ lymphocytes along gp120 gradients, leading these cells towards sources of HIV virions, and potentially increasing the likelihood of their infection (Kitchen *et al.*, 2002). Thus it appears that expression of CD4 on CD8 lymphocytes may generate cells with the cytotoxic ability of CD8 cells but the migration patterns of CD4 lymphocytes.

6.3.1.4 **Consequences of HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes at a cellular level.**

In order for HIV infection of CD8 lymphocytes to contribute directly to immunodeficiency, HIV infection must either kill the infected cell or impair its function. HIV infection has been shown to be cytotoxic to CD8 lymphocytes *in vitro* (Zerhouni *et al.*, 2004), and infection of *in vitro* generated $CD8^{bright}CD4^{dim}$ lymphocytes led to downregulation of CD4 expression (leading to impaired control of IFN-γ and Fas-L production), and impaired cytotoxicity (Kitchen *et al.*, 2004).

6.3.1.5 **Summary of section 6.3.1**

In summary, the available evidence demonstrates that $CD8^{bright}CD4^{dim}$ lymphocytes are functioning cells with distinct migration and effector characteristics, and suggests that the level of HIV infection of these cells observed in blood may belie a much greater level of infection in tissues. Thus HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes could have a significant impact on the CD8 lymphocyte response to acute and chronic pathogens.

6.3.2 CD8 lymphocytes as a reservoir of HIV infection.

As described in section 1.9, quiescent CD4 lymphocytes are currently thought be the longest lived HIV infected cells, forming a reservoir of provirus which is hidden from antiretroviral and immune attack. These quiescent CD4 lymphocytes can either be naïve lymphocytes infected during regenerative proliferation (Brenchley *et al.*, 2004a), or antigen experienced cells that were infected during activation but subsequently return to a quiescent state as long lived memory cells. The findings presented in chapter 5 show that the majority of circulating $CD8^{bright}CD4^{dim}$ lymphocytes have a memory phenotype and do not display activation markers, suggesting that $CD8^{bright}CD4^{dim}$ lymphocytes can similarly 'rest down' following antigen specific activation. In general CD8 lymphocytes are much more likely to 'rest down' than CD4 lymphocytes (Homann *et al.*, 2001; Foulds *et al.*, 2002), making them a more efficient conduit for generation of a viral reservoir.

As yet there is no direct evidence that quiescent CD8 lymphocytes are infected, but data showing that the CD8 lymphocyte proviral load was stable over months of follow up, and the detection of ancestral HIV variants in CD8 lymphocytes both suggest infection of a longlived population (McBreen *et al.*, 2001; Potter *et al.*, 2003).

6.3.3 CD8 lymphocytes as a source of virus.

As demonstrated in chapter 4, in subjects with advanced HIV disease CD8 lymphocytes contribute a substantial proportion (approximately 25%) of circulating proviral load. This finding is in keeping with results reported by other investigators.

If the proportions seen in blood are reflected in the total lymphocyte population, and if these infected CD8 lymphocytes generate progeny virions at rates similar to (or greater than) CD4 lymphocytes, then CD8 lymphocytes would represent a significant source of new virus. As discussed above the little data available supports greater levels of HIV infection of CD8 lymphocytes in the tissues than in the blood.

As discussed in section 1.10, although HIV replication has not been observed in CD8 lymphocytes *in vivo*, productive infection has been amply demonstrated *in vitro* both by staining for p24 and detection of HIV budding from CD8 lymphocytes by electron microscopy (Flamand *et al.*, 1998). Phylogenetic studies have the potential to demonstrate the degree to which a proviral population (such as HIV infected CD8 lymphocytes) contributes to the circulating viraemia, but these studies have shown marked interpatient variability (see section 1.11.3). Thus Potter *et al.*, (2003), report that in two of four subjects assessed, CD8 lymphocyte sequences were closely related to plasma sequences (suggesting either recent infection of the CD8 lymphocytes or productive infection of CD8 lymphocytes), but in the other two the plasma and CD8 lymphocyte derived sequences were segregated. Of interest different cell types do have marked variation in their efficiency of virion generation, for example, while monocytes are infected with HIV at relatively low frequency (McElrath *et al.*, 1991), they can contribute greater than 10% of the systemic viral load, with their contribution increasing during opportunistic infection (Lawn *et al.*, 2000).

6.4 Future Direction

The data presented in this thesis, together with an increasing body of published data, has identified $CD8^{bright}CD4^{dim}$ lymphocytes as cells with a major role in HIV immunopathogenesis. Future research should focus on improving our understanding of the natural history of these cells, and determining the extent to which there infection can influence disease progression or response to treatment.

There has been extensive characterisation of phenotype and function of *in vitro* generated $CD8^{bright}CD4^{dim}$ lymphocytes, and research could usefully refocus on the situation *in vivo*. Perhaps the most pressing questions regard the extent of CD4

upregulation in lymph nodes and inflamed tissues, especially during acute infection. Also of interest would be elucidation of the circumstances resulting in CD4 upregulation, for example, whether CD4 is upregulated during regenerative proliferation or only on antigen specific activation, and whether activation of memory as well as naïve CD8 lymphocytes results in CD4 expression.

The question of the relevance of HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes to disease progression, would be furthered by investigation of the extent of HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes in lymph node and solid tissues, and confirmation that infection is productive. Conclusive evidence that infection of $CD8^{bright}CD4^{dim}$ lymphocytes has a role in CD8 lymphocyte dysfunction will be difficult to generate, and whether yet another study of correlates of disease progression would be particularly informative is debatable.

The influence of therapeutics on HIV infected CD8 lymphocytes is of clear importance, both in terms of penetration of antiretrovirals, and effects of immune stimulants. To date zidovudine has been shown to be active against HIV infecting CD8 lymphocytes (Mercure *et al.*, 1994), but to my knowledge other antiretrovirals have not been specifically tested in these cells. Also of concern is the effect of immune stimulants such as hydroxyurea and IL-2. It is conceivable that these interventions may increase CD4 expression on CD8 lymphocytes thus providing further targets for HIV to infect.

Abreviations List

AIDS	Acquired Immunodeficiency Syndrome
ACP	Allophycocyanin
APC	Antigen presenting cell
CAF	CD8 cell antiviral factor
CCR	CC chemokine receptor
CD	Cluster of differentiation
CMV	cytomegalovirus
CNS	central nervous system
CTL	Cytotoxic T lymphocyte
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing non-integrin
DNA	DeoxyriboNucleic Acid
EBV	Epstein-Barr Virus
Env	Envelope gene
FACS	Fluorescent activated cell sorting
FITC	fluoroscein isothyocyanate
FSC	Forward scatter
Gag	Group specific antigen gene
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
LTR	Long terminal repeat
LTNP	Long term non-progressor
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
MACS	Magnetic activated cell sorting
MHC	Major histocompatibility complex
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NSI	Non-syncytium inducing
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohaemaglutinin
PHI	Primary HIV infection
PIC	Pre-integration complex
Pol	polymerase gene
Rev	Regulator of viral protein expression
RNA	RiboNucleic Acid
RT	Reverse transcriptase
RTC	Reverse transcription complex

SI	Syncytium inducing
SIV	Simian Immunodeficiency Virus
SHIV	SIV/HIV fusion virus
SSC	Side scatter
SEB	Staphylococcal enterotoxin B
STI	Structured treatment interruption
Tat	Retroviral transactivator of transcription
TNF	Tumor necrosis factor
Vif	Viral infectivity factor
Vpr	viral protein R
Vpu	viral protein U
Vpx	viral protein X

Reference List

Agostini, C. & Semenzato, G. (2002). Why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection. *Blood* **99**, 1876-1877.

Agy, M. B., Wambach, M., Foy, K. & Katze, M. G. (1990). Expression of cellular genes in CD4 positive lymphoid cells infected by the human immunodeficiency virus, HIV-1: evidence for a host protein synthesis shut-off induced by cellular mRNA degradation. *Virology* **177**, 251-258.

Allen, T. M. & Watkins, D. I. (2001). New insights into evaluating effective T-cell responses to HIV. *AIDS* **15 Suppl 5:S117-26.**, S117-S126.

Altfeld, M., Allen, T. M., Yu, X. G., Johnston, M. N., Agrawal, D., Korber, B. T., Montefiori, D. C., O'Connor, D. H., Davis, B. T., Lee, P. K., Maier, E. L., Harlow, J., Goulder, P. J., Brander, C., Rosenberg, E. S. & Walker, B. D. (2002). HIV-1 superinfection despite broad CD8+ T-cell responses containing replication of the primary virus. *Nature* **420**, 434-439.

Altfeld, M. & Rosenberg, E. S. (2000). The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1. *Curr Opin Immunol* **12**, 375-380.

Altman, J. D. & Feinberg, M. B. (2004). HIV escape: there and back again. *Nat Med* **10**, 229-230.

Altman, J. D., Moss, P. A. H., Goulder, P. J. R., Barouch, D. H., McHeyzerWilliams, M. G., Bell, J. I., McMichael, A. J. & Davis, M. M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**, 94-96.

Andersson, J., Kinloch, S., Sonnerborg, A., Nilsson, J., Fehniger, T. E., Spetz, A. L., Behbahani, H., Goh, L. E., Mcdade, H., Gazzard, B., Stellbrink, H., Cooper, D. & Perrin, L. (2002). Low levels of perforin expression in CD8+ T lymphocyte granules in lymphoid tissue during acute human immunodeficiency virus type 1 infection. *J Infect Dis* **185**, 1355-1358.

Angel, J. B., Kumar, A., Parato, K., Filion, L. G., Diazmitoma, F., Daftarian, P., Pham, B., Sun, E., Leonard, J. M. & Cameron, D. W. (1998). Improvement in cell-mediated immune function during potent anti- human immunodeficiency virus therapy with ritonavir plus saquinavir. *J Infect Dis* **177**, 898-904.

Anton, P. A., Mitsuyasu, R. T., Deeks, S. G., Scadden, D. T., Wagner, B., Huang, C., Macken, C., Richman, D. D., Christopherson, C., Borellini, F., Lazar, R. & Hege, K. M. (2003). Multiple measures of HIV burden in blood and tissue are correlated with each other but not with clinical parameters in aviremic subjects. *AIDS* **17**, 53-63.

Appay, V., Dunbar, P. R., Callan, M., Klenerman, P., Gillespie, G. M., Papagno, L., Ogg, G. S., King, A., Lechner, F., Spina, C. A., Little, S., Havlir, D. V., Richman, D. D., Gruener, N., Pape, G., Waters, A., Easterbrook, P., Salio, M., Cerundolo, V., McMichael, A. J. & Rowland-Jones, S. L. (2002a). Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* **8**, 379-385.

Appay, V., Nixon, D. F., Donahoe, S. M., Gillespie, G. M., Dong, T., King, A., Ogg, G. S., Spiegel, H. M., Conlon, C., Spina, C. A., Havlir, D. V., Richman, D. D., Waters, A., Easterbrook, P., McMichael, A. J. & Rowland-Jones, S. L. (2000). HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J Exp Med* **192**, 63-75.

Appay, V. & Rowland-Jones, S. L. (2002). Premature ageing of the immune system: the cause of AIDS? *Trends Immunol* **23**, 580-585.

Appay, V., Zaunders, J. J., Papagno, L., Sutton, J., Jaramillo, A., Waters, A., Easterbrook, P., Grey, P., Smith, D., McMichael, A. J., Cooper, D. A., Rowland-Jones, S. L. & Kelleher, A. D. (2002b). Characterization of CD4(+) CTLs ex vivo. *J Immunol* **168**, 5954-5958.

Arno, A., Ruiz, L., Juan, M., Jou, A., Balague, M., Zayat, M. K., Marfil, S., MartinezPicado, J., Martinez, M. A., Romeu, J., PujolBorrell, R., Lane, C. & Clotet, B. (1999). Efficacy of low-dose subcutaneous interleukin-2 to treat advanced human immunodeficiency virus type 1 in persons with <= 250/mu L CD4 T cells and undetectable plasma virus load. *J Infec Dis* **180**, 56-60.

Autran, B., Carcelain, G., Li, T. S., Blanc, C., Mathez, D., Tubiana, R., Katlama, C., Debre, P. & Leibowitch, J. (1997). Positive effects of combined antiretroviral therapy on CD4(+) T cell homeostasis and function in advanced HIV disease. *Science* **277**, 112-116.

Bagot, M., Echchakir, H., Mami-Chouaib, F., Delfau-Larue, M. H., Charue, D., Bernheim, A., Chouaib, S., Boumsell, L. & Bensussan, A. (1998). Isolation of tumor-specific cytotoxic CD4+ and CD4+CD8dim+ T-cell clones infiltrating a cutaneous T-cell lymphoma. *Blood* **91**, 4331-4341.

Bandres, J. C., Wang, Q. F., O'Leary, J., Baleaux, F., Amara, A., Hoxie, J. A., Zolla-Pazner, S. & Gorny, M. K. (1998). Human immunodeficiency virus (HIV) envelope binds to CXCR4

independently of CD4, and binding can be enhanced by interaction with soluble CD4 or by HIV envelope deglycosylation. *J Virol* **72**, 2500-2504.

Barre Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler Blin, C., Vezinet Brun, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L. (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**, 868-871.

Bell, E. B. & Sparshott, S. M. (1990). Interconversion of CD45R subsets of CD4 T cells in vivo. *Nature* **348**, 163-166.

Bell, J. E., Busuttil, A., Ironside, J. W., Rebus, S., Donaldson, Y. K., Simmonds, P. & Peutherer, J. F. (1993). Human immunodeficiency virus and the brain - investigation of virus load and neuropathologic changes in pre-AIDS subjects. *J Infect Dis* **168**, 818-824.

Benito, J. M., Lopez, M., Lozano, S., Martinez, P., Gonzalez-Lahoz, J. & Soriano, V. (2004). CD38 expression on CD8 T lymphocytes as a marker of residual virus replication in chronically HIV-infected patients receiving antiretroviral therapy. *AIDS Res Hum Retroviruses* **20**, 227-233.

Benito, J. M., Zabay, J. M., Gil, J., Bermejo, M., Escudero, A., Sanchez, E. & Fernandez-Cruz, E. (1997). Quantitative alterations of the functionally distinct subsets of CD4 and CD8 T lymphocytes in asymptomatic HIV infection: changes in the expression of CD45RO, CD45RA, CD11b, CD38, HLA-DR, and CD25 antigens. *J Acquir Immune Defic Syndr Hum Retrovirol* **14**, 128-135.

Bharadwaj, M., Burrows, S. R., Burrows, J. M., Moss, D. J., Catalina, M. & Khanna, R. (2001). Longitudinal dynamics of antigen-specific CD8+ cytotoxic T lymphocytes following primary Epstein-Barr virus infection. *Blood* **98**, 2588-2589.

Blaak, H., van't Wout, A. B., Brouwer, M., Hooibrink, B., Hovenkamp, E. & Schuitemaker, H. (2000). In vivo HIV-1 infection of CD45RA(+)CD4(+) T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4(+) T cell decline. *Proc Natl Acad Sci U S A* **97**, 1269-1274.

Blue, M. L., Daley, J. F., Levine, H. & Schlossman, S. F. (1985). Coexpression of T4 and T8 on peripheral blood T cells demonstrated by two-color fluorescence flow cytometry. *J Immunol* **134**, 2281-2286.

Blumberg, R. S., Paradis, T., Byington, R., Henle, W., Hirsch, M. S. & Schooley, R. T. (1987). Effects of human immunodeficiency virus on the cellular immune response to Epstein-Barr virus in homosexual men: characterization of the cytotoxic response and lymphokine production. *J Infect Dis* **155**, 877-890.

Boni, C., Bertoletti, A., Penna, A., Cavalli, A., Pilli, M., Urbani, S., Scognamiglio, P., Boehme, R., Panebianco, R., Fiaccadori, F. & Ferrari, C. (1998). Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. *J Clin Invest* **102**, 968-975.

Bonomo, A., Kehn, P. J. & Shevach, E. M. (1994). Premature escape of double-positive thymocytes to the periphery of young mice. Possible role in autoimmunity. *J Immunol* **152**, 1509-1514.

Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M. & Oldstone, M. B. A. (1994). Virus-specific CD8+ cytotoxic t-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* **68**, 6103-6110.

Borrow, P., Lewicki, H., Wei, X. P., Horwitz, M. S., Peffer, N., Meyers, H., Nelson, J. A., Gairin, J. E., Hahn, B. H., Oldstone, M. B. A. & Shaw, G. M. (1997). Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nature Med* **3**, 205-211.

Boyer, J. D., Cohen, A. D., Vogt, S., Schumann, K., Nath, B., Ahn, L., Lacy, K., Bagarazzi, M. L., Higgins, T. J., Baine, Y., Ciccarelli, R. B., Ginsberg, R. S., Macgregor, R. R. & Weiner, D. B. (2000). Vaccination of seronegative volunteers with a human immunodeficiency virus type 1 env/rev DNA vaccine induces antigen-specific proliferation and lymphocyte production of beta-chemokines. *J Infect Dis* **181**, 476-483.

Boyer, V., Smith, L. R., Ferre, F., Pezzoli, P., Trauger, R. J., Jensen, F. C. & Carlo, D. J. (1993). T-cell receptor v-beta repertoire in HIV-infected individuals - lack of evidence for selective v-beta deletion. *Clin Exp Immunol* **92**, 437-441.

Brenchley, J. M., Hill, B. J., Ambrozak, D. R., Price, D. A., Guenaga, F. G., Casazza, J. P., Kuruppu, J., Yazdani, J., Migueles, S. A., Connors, M., Roederer, M., Douek, D. C. & Koup, R. A. (2004a). T-cell subsets that harbour human immunodeficiency virus (HIV) in vivo: implications for HIV pathogenesis. *Journal of Virology* **78**, 1160-1168.

Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., Nguyen, P. L., Khoruts, A., Larson, M., Haase, A. T. & Douek, D. C. (2004b). CD4+ T cell

depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* **200**, 749-759.

Brites, C., Alencar, R., Gusmao, R., Pedroso, C., Netto, E. M., Pedral-Sampaio, D. & Badaro, R. (2001). Co-infection with HTLV-1 is associated with a shorter survival time for HIV-1-infected patients in Bahia, Brazil. *AIDS* **15**, 2053-2055.

Brites, C., Harrington, W., Jr., Pedroso, C., Martins, N. E. & Badaro, R. (1997). Epidemiological Characteristics of HTLV-I and II Co-Infection in Brazilian Subjects Infected by HIV-1. *Braz J Infect Dis* **1**, 42-47.

Brites, C., Pedroso, C., Netto, E., Harrington, W., Jr., Galvao-Castro, B., Couto-fernandez, J. C., Pedral-Sampaio, D., Morgado, M., Teixeira, R. & Badaro, R. (1998). Co-Infection by HTLV-I/II is Associated With Increased Viral Load in PBMC of HIV-1 Infected Patients in Bahia, Brazil. *Braz J Infect Dis* **2**, 70-77.

Brooks, D. G., Kitchen, S. G., Kitchen, C. M., Scripture-Adams, D. D. & Zack, J. A. (2001). Generation of HIV latency during thymopoiesis. *Nat Med* **7**, 459-64.

Broussard, S. R., Staprans, S. I., White, R., Whitehead, E. M., Feinberg, M. B. & Allan, J. S. (2001). Simian immunodeficiency virus replicates to high levels in naturally infected African green monkeys without inducing immunologic or neurologic disease. *J Virol* **75**, 2262-2275.

Bucy, R. P., Hockett, R. D., Derdeyn, C. A., Saag, M. S., Squires, K., Sillers, M., Mitsuyasu, R. T. & Kilby, J. M. (1999). Initial increase in blood CD4(+) lymphocytes after HIV antiretroviral therapy reflects redistribution from lymphoid tissues. *J Clin Invest* **103**, 1391-1398.

Bukrinsky, M., Sharova, N. & Stevenson, M. (1993). Human immunodeficiency virus type-1 2-LTR circles reside in a nucleoprotein complex which is different from the preintegration complex. *J Virol* **67**, 6863-6865.

Bunce, C. & Bell, E. B. (1997) . CD45RC isoforms define two types of CD4 memory T cells, one of which depends on persisting antigen. *J Exp Med* **185**, 767-776.

Burke, A. P., Anderson, D., Benson, W., Turnicky, R., Mannan, P., Liang, Y. H., Smialek, J. & Virmani, R. (1995). Localization of human immunodeficiency virus 1 RNA in thymic tissues from asymptomatic drug addicts. *Arch Pathol Lab Med* **119**, 36-41.

Busch, M. P., Lee, L. L. L., Satten, G. A., Henrard, D. R., Farzadegan, H., Nelson, K. E., Read, S., Dodd, R. Y. & Petersen, L. R. (1995). Time course of detection of viral and serologic markers preceding human immunodeficiency virus type 1 seroconversion: implications for screening of blood and tissue donors. *Transfusion* **35**, 91-97.

Cai, Q., Huang, X. L., Rappocciolo, G. & Rinaldo, C. R., Jr. (1990). Natural killer cell responses in homosexual men with early HIV infection. *J Acquir Immune Defic Syndr* **3**, 669-676.

Calabro, M. L., Zanotto, C., Calderazzo, F., Crivellaro, C., Delmistro, A., Derossi, A. & Chiecobianchi, L. (1995). HIV-1 infection of the thymus: evidence for a cytopathic and thymotropic viral variant in vivo. *AIDS Res Hum Retroviruses* **11**, 11-19.

Callan, M. F. C., Steven, J., Krausa, P., Wilson, J. D. K., Moss, P. A. H., Gillespie, G. M., Bell, J. I., Rickinson, A. B. & McMichael, A. J. (1996). Large clonal expansions of CD8(+) T cells in acute infectious mononucleosis. *Nature Med* **2**, 906-911.

Cardin, R. D., Brooks, J. W., Sarawar, S. R. & Doherty, P. C. (1996). Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. *J Exp Med* **184**, 863-871.

Carmichael, A., Jin, X., Sissons, P. & Borysiewicz, L. (1993). Quantitative analysis of the human immunodeficiency virus type-1 (HIV-1)-specific cytotoxic lymphocyte-t (CTL) response at different stages of HIV-1 infection - differential CTL responses to HIV-1 and epstein-barr virus in late disease. *J Exp Med* **177**, 249-256.

Carney, W. P. & Hirsch, M. S. (1981). Mechanisms of immunosuppression in cytomegalovirus mononucleosis. II. Virus-monocyte interactions. *J Infect Dis* **144**, 47-54.

Castro, K. G., Ward, J. W., Slutsker, L., Buehler, J. W., Jaffe, H. W. & Berkelman, R. L. (1992). 1993 Revised Classification System for HIV Infection and Expanded Surveillance Case Definition for AIDS Among Adolescents and Adults. **41(RR-17)**.

Center, D. M., Kornfeld, H., Ryan, T. C. & Cruikshank, W. W. (2000). Interleukin 16: implications for CD4 functions and HIV-1 progression. *Immunol Today* **21**, 273-280.

Chakrabarti, L. A., Lewin, S. R., Zhang, L., Gettie, A., Luckay, A., Martin, L. N., Skulsky, E., Ho, D. D., Cheng-Mayer, C. & Marx, P. A. (2000). Normal T-cell turnover in sooty mangabeys harboring active simian immunodeficiency virus infection. *J Virol* **74**, 1209-1223.

Champagne, P., Ogg, G. S., King, A. S., Knabenhans, C., Ellefsen, K., Nobile, M., Appay, V., Rizzardi, G. P., Fleury, S., Lipp, M., Forster, R., Rowland-Jones, S., Sekaly, R. P., McMichael, A. J. & Pantaleo, G. (2001). Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* **410**, 106-111.

Chan, D. C., Fass, D., Berger, J. M. & Kim, P. S. (1997). Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**, 263-273.

Chanh, T. C., Kennedy, R. C. & Kanda, P. (1988). Synthetic peptides homologous to HIV transmembrane glycoprotein suppress normal human lymphocyte blastogenic response. *Cell Immunol* **111**, 77-86.

Chen, G., Shankar, P., Lange, C., Valdez, H., Skolnik, P. R., Wu, L., Manjunath, N. & Lieberman, J. (2001). CD8 T cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection. *Blood* **98**, 156-164.

Chun, T. W., Carruth, L., Finzi, D., Shen, X. F., DiGiuseppe, J. A., Taylor, H., Hermankova, M., Chadwick, K., Margolick, J., Quinn, T. C., Kuo, Y. H., Brookmeyer, R., Zeiger, M. A., BarditchCrovo, P. & Siliciano, R. F. (1997). Quantification of latent tissue reservoirs and total body viral load in HIV-1 Infection. *Nature* **387**, 183-188.

Chun, T. W., Finzi, D., Margolick, J., Chadwick, K., Schwartz, D. & Siliciano, R. F. (1995). In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat Med* **1**, 1284-1290.

Churdboonchart, V., Moss, R. B., Sirawaraporn, W., Smutharaks, B., Sutthent, R., Jensen, F. C., Vacharak, P., Grimes, J., Theofan, G. & Carlo, D. J. (1998). Effect of HIV-specific immune-based therapy in subjects infected with HIV-1 subtype E in Thailand. *AIDS* **12**, 1521-1527.

Cleland, A., Davis, C., Adams, N., Lycett, C., Jarvis, L. M., Holmes, H. & Simmonds, P. (2001). Development of multiplexed nucleic acid testing for human immunodeficiency virus type 1 and hepatitis C virus. *Vox Sang* **81** , 93-101.

Clerici, M., Hakim, F. T., Venzon, D. J., Blatt, S., Hendrix, C. W., Wynn, T. A. & Shearer, G. M. (1993). Changes in interleukin-2 and interleukin-4 production in asymptomatic, human immunodeficiency virus-seropositive individuals. *J Clin Invest* **91**, 759-765.

Clerici, M., Landay, A. L., Kessler, H. A., Zajac, R. A., Boswell, R. N., Muluk, S. C. & Shearer, G. M. (1991) . Multiple patterns of alloantigen presenting/stimulating cell dysfunction in patients with AIDS. *J Immunol* **146**, 2207-2213.

Clerici, M. & Shearer, G. M. (1997). The Th1-Th2 hypothesis of HIV infection: new insights. *Immunology today* **15**, 575-581.

Clerici, M., Stocks, N. I., Zajac, R. A., Boswell, R. N., Lucey, D. R., Via, C. S. & Shearer, G. M. (1989). Detection of three distinct patterns of T helper cell dysfunction in asymptomatic human immunodeficiency virus-seropositive patients. Independence of CD4+ numbers and clinical staging. *J Clin Invest* **84**, 1892-1899.

Cloyd, M. & Lynn, W. S. (1991) . Perturbation of host-cell membrane is a primary mechanism of HIV cytopathology. *Virology* **181**, 500-511.

Craston, R., Koh, M., Mc, D. A., Ray, N., Prentice, H. G. & Lowdell, M. W. (1997). Temporal dynamics of CD69 expression on lymphoid cells. *J Immunol Methods* **209**, 37-45.

Dalod, M., Dupuis, M., Deschemin, J. C., Sicard, D., Salmon, D., Delfraissy, J. F., Venet, A., Sinet, M. & Guillet, J. G. (1999). Broad, intense anti-human immunodeficiency virus (HIV) ex vivo CD8(+) responses in HIV type 1-infected patients: Comparison with anti- Epstein-Barr virus responses and changes during antiretroviral therapy. *J Virol* **73**, 7108-7116.

Dalod, M., Fiorentino, S., Delamare, C., Rouzioux, C., Sicard, D., Guillet, J. G. & Gomard, E. (1996). Delayed virus-specific CD8(+) cytotoxic T lymphocyte activity in an HIV-infected individual with high CD4(+) cell counts: Correlations with various parameters of disease progression. *AIDS Res Hum Retroviruses* **12**, 497-506.

Davis, L. E., Hjelle, B. L. & Miller, V. E. (1992). Early brain invasion in iatrogenic human immunodeficiency virus infection. *Neurology* **42**, 1736-1739.

de Araujo, A. C., Casseb, J. S., Neitzert, E., de Souza, M. L., Mammano, F., Del Mistro, A., De Rossi, A. & Chieco-Bianchi, L. (1994). HTLV-I and HTLV-II infections among HIV-1 seropositive patients in Sao Paulo, Brazil. *Eur J Epidemiol* **10**, 165-171.

De Maria, A., Colombini, S., Schnittman, S. & Moretta, L. (1994). CD8+ cytolytic T lymphocytes become infected in vitro in the process of killing HIV-1-infected target cells. *Eur J Imunol* **24**, 531-536.

De Maria, A., Mavilio, D., Costa, P., Dignetti, P., Fogli, M. & Mingari, M. C. (2000). Multiple HLA-class I-specific inhibitory NK receptor expression and IL-4/IL-5 production by CD8+ T-cell clones in HIV-1 infection. *Immunol Lett* **72**, 179-182.

De Maria, A., Pantaleo, G., Schnittman, S. M., Greenhouse, J. J., Baseler, M., Orenstein, J. M. & Fauci, A. S. (1991). Infection of CD8+ T lymphocytes with HIV. Requirements for interaction with infected CD4+ cells and induction of infectious virus from chronically infected CD8+ cells. *J Immunol* **146**, 2220-2226.

De Rosa, S. C., Herzenberg, L. A., Herzenberg, L. A. & Roederer, M. (2001). 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. *Nat Med* **7**, 245-248.

De Rossi, A., Calabro, M. L., Panozzo, M., Bernardi, D., Caruso, B., Tridente, G. & Chieco-Bianchi, L. (1990). In vitro studies of HIV-1 infection in thymic lymphocytes: a putative role of the thymus in AIDS pathogenesis. *AIDS Res Hum Retroviruses* **6**, 287-298.

Deacon, N. J., Tsykin, A., Solomon, A., Smith, K., Ludfordmenting, M., Hooker, D. J., McPhee, D. A., Greenway, A. L., Ellett, A., Chatfield, C., Lawson, V. A., Crowe, S., Maerz, A., Sonza, S., Learmont, J., Sullivan, J. S., Cunningham, A., Dwyer, D., Dowton, D. & Mills, J. (1995). Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* **270**, 988-991.

Dean, G. A., Reubel, G. H. & Pedersen, N. C. (1996). Simian immunodeficiency virus infection of CD8+ lymphocytes in vivo. *J Virol* **70**, 5646-5650.

Demaria, A., Ferraris, A., Guastella, M., Pilia, S., Cantoni, C., Polero, L., Mingari, M. C., Bassetti, D., Fauci, A. S. & Moretta, L. (1997). Expression of HLA class I-specific inhibitory natural killer cell receptors in HIV-specific cytolytic T lymphocytes: Impairment of specific cytolytic functions. *Proc Natl Acad Sci USA* **94**, 10285-10288.

Diamond, D. C., Sleckman, B. P., Gregory, T., Lasky, L. A., Greenstein, J. L. & Burakoff, S. J. (1988). Inhibition of CD4+ T cell function by the HIV envelope protein, gp120. *J Immunol* **141**, 3715-3717.

Doisne, J. M., Urrutia, A., Lacabaratz-Porret, C., Goujard, C., Meyer, L., Chaix, M. L., Sinet, M. & Venet, A. (2004). CD8+ T cells specific for EBV, cytomegalovirus, and influenza virus are activated during primary HIV infection. *J Immunol* **173**, 2410-2418.

Doms, R. W. & Trono, D. (2000) . The plasma membrane as a combat zone in the HIV battlefield. *Genes Dev* **14**, 2677-2688.

Douek, D. C., Brenchley, J. M., Betts, M. R., Ambrozak, D. R., Hill, B. J., Okamoto, Y., Casazza, J. P., Kuruppu, J., Kunstman, K., Wolinsky, S., Grossman, Z., Dybul, M., Oxenius, A., Price, D. A., Connors, M. & Koup, R. A. (2002). HIV preferentially infects HIV-specific CD4+ T cells. *Nature* **417** , 95-98.

Douek, D. C., Picker, L. J. & Koup, R. A. (2003). T cell dynamics in HIV-1 infection. *Annu Rev Immunol* **21**, 265-304.

Douglas, M. P. & Rogers, S. O. (1998). DNA damage caused by common cytological fixatives. *Mutat Res* **401**, 77-88.

Dudhane, A., Conti, B., Orlikowsky, T., Wang, Z. Q., Mangla, N., Gupta, A., Wormser, G. P. & Hoffmann, M. K. (1996). Monocytes in HIV type 1-infected individuals lose expression of costimulatory B7 molecules and acquire cytotoxic activity. *AIDS Res Hum Retroviruses* **12**, 885-892.

Dumonceaux, J., Nisole, S., Chanel, C., Quivet, L., Amara, A., Baleux, F., Briand, P. & Hazan, U. (1998). Spontaneous mutations in the env gene of the human immunodeficiency virus type 1 NDK isolate are associated with a CD4-independent entry phenotype. *J Virol* **72**, 512-519.

Dybul, M., Nies-Kraske, E., Daucher, M., Hertogs, K., Hallahan, C. W., Csako, G., Yoder, C., Ehler, L., Sklar, P. A., Belson, M., Hidalgo, B., Metcalf, J. A., Davey, R. T., Rock Kress, D. M., Powers, A. & Fauci, A. S. (2003). Long-cycle structured intermittent versus continuous highly active antiretroviral therapy for the treatment of chronic infection with human immunodeficiency virus: effects on drug toxicity and on immunologic and virologic parameters. *J Infect Dis* **188**, 388-396.

Edinger, A. L., Mankowski, J. L., Doranz, B. J., Margulies, B. J., Lee, B., Rucker, J., Sharron, M., Hoffman, T. L., Berson, J. F., Zink, M. C., Hirsch, V. M., Clements, J. E. & Doms, R. W. (1997). CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain. *Proc Natl Acad Sci U S A* **94**, 14742-14747.

Eggena, M. P., Barugahare, B., Okello, M., Mutyala, S., Jones, N., Ma, Y., Kityo, C., Mugyenyi, P. & Cao, H. (2005). T cell activation in HIV-seropositive Ugandans: differential associations with viral load, CD4+ T cell depletion, and coinfection. *J Infect Dis* **191**, 694-701.

Ekong, T., Gompels, M., Clark, C., Parkin, J. & Pinching, A. (1993). Double-staining artefact observed in certain individuals during dual-colour immunophenotyping of lymphocytes by flow cytometry. *Cytometry* **14**, 679-684.

Endres, M. J., Clapham, P. R., Marsh, M., Ahuja, M., Davis Turner, J., McKnight, A., Thomas, J. F., Stoebenau-Haggarty, B., Choe, S., Vance, P. J., Wells, T. N. C., Power, C. A., Sutterwalla, S. S., Doms, R. W., Landau, N. R. & Hoxie, J. A. (1996). CD4-independent infection by HIV-2 is mediated by fusin/CXCR4. *Cell* **87**, 745-756.

Epstein, J. S., Frederick, W. R., Rook, A. H., Jackson, L., Manischewitz, J. F., Mayner, R. E., Masur, H., Enterline, J. C., Djeu, J. Y. & Quinnan, G. V., Jr. (1985). Selective defects in cytomegalovirus- and mitogen-induced lymphocyte proliferation and interferon release in patients with acquired immunodeficiency syndrome. *J Infect Dis* **152**, 727-733.

Finzi, D., Blankson, J., Siliciano, J. D., Margolick, J. B., Chadwick, K., Pierson, T., Smith, K., Lisziewicz, J., Lori, F., Flexner, C., Quinn, T. C., Chaisson, R. E., Rosenberg, E., Walker, B., Gange, S., Gallant, J. & Siliciano, R. F. (1999). Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med* **5**, 512-517.

Finzi, D., Hermankova, M., Pierson, T., Carruth, L. M., Buck, C., Chaisson, R. E., Quinn, T. C., Chadwick, K., Margolick, J., Brookmeyer, R., Gallant, J., Markowitz, M., Ho, D. D., Richman, D. D. & Siliciano, R. F. (1997). Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* **278**, 1295-1300.

Flamand, L., Crowley, R. W., Lusso, P., Colombini-Hatch, S., Margolis, D. & Gallo, R. C. (1997). CD4-mediated infection of primary CD8+ lymphocytes by human immunodeficienc virus. *in press*.

Flamand, L., Crowley, R. W., Lusso, P., ColombiniHatch, S., Margolis, D. M. & Gallo, R. C. (1998). Activation of CD8(+) T lymphocytes through the T cell receptor turns on CD4 gene expression: Implications for HIV pathogenesis. *Proc Natl Acad Sci USA* **95**, 3111-3116.

Foulds, K. E., Zenewicz, L. A., Shedlock, D. J., Jiang, J., Troy, A. E. & Shen, H. (2002). Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. *J Immunol* **168**, 1528-1532.

Franz, J. K., Kolb, S. A., Hummel, K. M., Lahrtz, F., Neidhart, M., Aicher, W. K., Pap, T., Gay, R. E., Fontana, A. & Gay, S. (1998). Interleukin-16, produced by synovial fibroblasts, mediates chemoattraction for CD4+ T lymphocytes in rheumatoid arthritis. *Eur J Immunol* **28**, 2661-2671.

Frederick, W., Massur, H., Rook, AH., Mittal, K., Manischewitz, J., Jackson, L., Staus, s. & Quinnan, g. (1982). Immune functions during cytomegalovirus infection in immunodeficient male homosexuals. *Sixth Cold Spring Harbour meeting on Herpesvirus*.

French, M. A., Price, P. & Stone, S. F. (2004). Immune restoration disease after antiretroviral therapy. *AIDS* **18**, 1615-1627.

Galiani, M. D., Aguado, E., Tarazona, R., Romero, P., Molina, I., Santamaria, M., Solana, R. & Pena, J. (1999) . Expression of killer inhibitory receptors on cytotoxic cells from HIV- 1-infected individuals. *Clin Exp Immunol* **115**, 472-476.

Gallo, R. C., Sarin, P. S., Gelmann, E. P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V. S., Mann, D., Sidhu, G. D., Stahl, R. E., Zolla-Pazner, S., Leibowitch, J. & Popovic, M. (1983). Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* **220**, 865-867.

Gamadia, L. E., ten Berge, I. J., Picker, L. J. & van Lier, R. A. (2002). Skewed maturation of virus-specific CTLs? *Nat Immunol* **3**, 203.

Gamberg, J. C., Bowmer, M. I., Trahey, J. C., Campbell, C. M., Pardoe, I. & Grant, M. D. (1999). Functional and genetic integrity of the CD8 T-cell repertoire in advanced HIV infection. *AIDS* **13**, 2043-2053.

Gao, F., Bailes, E., Robertson, D. L., Chen, Y. L., Rodenburg, C. M., Michael, S. F., Cummins, L. B., Arthur, L. O., Peeters, M., Shaw, G. M., Sharp, P. M. & Hahn, B. H. (1999). Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397**, 436-441.

Gauduin, M. C., Parren, P. W. H. I., Weir, R., Barbas, C. F., Burton, D. R. & Koup, R. A. (1997). Passive immunization with a human monoclonal antibody protects hu-PBL- SCID mice against challenge by primary isolates of HIV-1. *Nature Med* **3**, 1389-1393.

Geginat, J., Lanzavecchia, A. & Sallusto, F. (2003). Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* **101**, 4260-4266.

Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., Kewalramani, V. N., Littman, D. R., Figdor, C. G. & van Kooyk, Y. (2000). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**, 587-597.

Geleziunas, R., Xu, W., Takeda, K., Ichijo, H. & Greene, W. C. (2001). HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature* **410**, 834-838.

Giorgi, J. V., Liu, Z., Hultin, L. E., Cumberland, W. G., Hennessey, K. & Detels, R. (1993). Elevated levels of CD38+ CD8+ T cells in HIV infection add to the prognostic value of low CD4+ T cell levels: results of 6 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study. *J Acquir Immune Defic Syndr* **6**, 904-912.

Goebel, F. D., Mannhalter, J. W., Belshe, R. B., Eibl, M. M., Grob, P. J., deGruttolae, V., Griffiths, P. D., Erfle, V., Kunschak, M. & Engl, W. (1999). Recombinant gp160 as a therapeutic vaccine for HIV-infection: results of a large randomized, controlled trial. *AIDS* **13**, 1461-1468.

Greene, W. C. & Peterlin, B. M. (2002). Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. *Nat Med* **8**, 673-680.

Grody, W. W., Fligiel, S. & Naeim, F. (1985). Thymus involution in the acquired immunodeficiency syndrome. *Am J Clin Pathol* **84**, 85-95.

Grossman, Z., Meier-Schellersheim, M., Sousa, A. E., Victorino, R. M. & Paul, W. E. (2002). CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause? *Nat Med* **8**, 319-323.

Gruters, R. A., Terpstra, F. G., De Jong, R., van Noesel, C. J., van Lier, R. A. & Miedema, F. (1990). Selective loss of T cell functions in different stages of HIV infection. Early loss of anti-CD3-induced T cell proliferation followed by decreased anti-CD3-induced cytotoxic T lymphocyte generation in AIDS-related complex and AIDS. *Eur J Immunol* **20**, 1039-1044.

Guadalupe, M., Reay, E., Sankaran, S., Prindiville, T., Flamm, J., Mcneil, A. & Dandekar, S. (2003). Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* **77**, 11708-11717.

Guimaraes, M. L., Bastos, F. I., Telles, P. R., Galvao-Castro, B., Diaz, R. S., Bongertz, V. & Morgado, M. G. (2001). Retrovirus infections in a sample of injecting drug users in Rio de Janeiro City, Brazil: prevalence of HIV-1 subtypes, and co-infection with HTLV-I/II. *J Clin Virol* **21**, 143-151.

Gurley, R. J., Ikeuchi, K., Byrn, R. A., Anderson, K. & Groopman, J. E. (1989). CD4+ lymphocyte function with early human immunodeficiency virus infection. *Proc Natl Acad Sci U S A* **86**, 1993-1997.

Hakim, F. T., Cepeda, R., Kaimei, S., Mackall, C. L., McAtee, N., Zujewski, J., Cowan, K. & Gress, R. E. (1997). Constraints on CD4 recovery postchemotherapy in adults: Thymic insufficiency and apoptotic decline of expanded peripheral CD4 cells. *Blood* **90**, 3789-3798.

Hamann, D., Baars, P., Rep, M. H. G., Hooibrink, B., KerkhofGarde, S. R., Klein, M. R. & Vanlier, R. A. W. (1997). Phenotypic and functional separation of memory and effector human CD8(+) T cells. *J Exp Med* **186**, 1407-1418.

Hamann, D., Kostense, S., Wolthers, K. C., Otto, S. A., Baars, P. A., Miedema, F. & van Lier, R. A. (1999a). Evidence that human CD8+CD45RA+CD27- cells are induced by antigen and evolve through extensive rounds of division. *Int Immunol* **11**, 1027-1033.

Hamann, D., Roos, M. T. & van Lier, R. A. (1999b). Faces and phases of human CD8 T-cell development. *Immunol Today* **20**, 177-180.

Hammer, S. M., Squires, K. E., Hughes, M. D., Grimes, J. M., Demeter, L. M., Currier, J. S., Eron, J. J., Feinberg, J. E., Balfour, H. H., Dayton, L. R., Chodakewitz, J. A. & Fischl, M. A. (1997). A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. *N Engl J Med* **337**, 725-733.

Harrer, T., Harrer, E., Kalams, S. A., Barbosa, P., Trocha, A., Johnson, R. P., Elbeik, T., Feinberg, M. B., Buchbinder, S. P. & Walker, B. D. (1996). Cytotoxic T lymphocytes in asymptomatic long-term nonprogressing HIV-1 infection - Breadth and specificity of the response and relation to in vivo viral quasispecies in a person with prolonged infection and low viral load. *J Immunol* **156**, 2616-2623.

Havlir, D. V., Schrier, R. D., Torriani, F. J., Chervenak, K., Hwang, J. Y. & Boom, W. H. (2000). Effect of potent antiretroviral therapy on immune responses to Mycobacterium avium in human immunodeficiency virus-infected subjects. *J Infect Dis* **182**, 1658-1663.

Haynes, B. F., Hale, L. P., Weinhold, K. J., Patel, D. D., Liao, H. X., Bressler, P. B., Jones, D. M., Demarest, J. F., GebhardMitchell, K., Haase, A. T. & Bartlett, J. A. (1999). Analysis of the adult thymus in reconstitution of T lymphocytes in HIV-1 infection. *J Clin Invest* **103**, 453-460.

Hazenberg, M. D., Otto, S. A., Cohen Stuart, J. W., Verschuren, M. C., Borleffs, J. C., Boucher, C. A., Coutinho, R. A., Lange, J. M., Rinke De Wit, T. F., Tsegaye, A., van Dongen, J. J., Hamann, D., de Boer, R. J. & Miedema, F. (2000a). Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. *Nat Med* **6**, 1036-1042.

Hazenberg, M. D., Stuart, J. W., Otto, S. A., Borleffs, J. C., Boucher, C. A., de Boer, R. J., Miedema, F. & Hamann, D. (2000b). T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). *Blood* **95**, 249-255.

Hellerstein, M., Hanley, M. B., Cesar, D., Siler, S., Papageorgopoulos, C., Wieder, E., Schmidt, D., Hoh, R., Neese, R., Macallan, D., Deeks, S. & McCune, J. M. (1999). Directly measured kinetics of circulating T lymphocytes in normal and HIV-1-infected humans. *Nature Med* **5**, 83-89.

Herbein, G., Mahlknecht, U., Batliwalla, F., Gregersen, P., Pappas, T., Butler, J., OBrien, W. A. & Verdin, E. (1998). Apoptosis of CD8(+) T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. *Nature* **395**, 189-194.

Hernandez, J., Garfias, Y., Nieto, A., Mercado, C., Montano, L. F. & Zenteno, E. (2001). Comparative evaluation of the CD4+CD8+ and CD4+CD8- lymphocytes in the immune response to porcine rubulavirus. *Vet Immunol Immunopathol* **79**, 249-259.

Hesselgesser, J., Halks-Miller, M., DelVecchio, V., Peiper, S. C., Hoxie, J., Kolson, D. L., Taub, D. & Horuk, R. (1997). CD4-independent association between HIV-1 gp120 and CXCR4: functional chemokine receptors are expressed in human neurons. *Curr Biol* **7**, 112-121.

Hirsch, M., Steigbigel, R., Staszewski, S., Mellors, J., Scerpella, E., Hirschel, B., Lange, J., Squires, K., Rawlins, S., Meibohm, A. & Leavitt, R. (1999a). A randomized, controlled trial of

indinavir, zidovudine, and lamivudine in adults with advanced human immunodeficiency virus type 1 infection and prior antiretroviral therapy. *J Infec Dis* **180**, 659-665.

Hirsch, V. M., Campbell, B. J., Bailes, E., Goeken, R., Brown, C., Elkins, W. R., Axthelm, M., Murpheycorb, M. & Sharp, P. M. (1999b). Characterization of a novel simian immunodeficiency virus (SIV) from L'Hoest monkeys (Cercopithecus l'hoesti): Implications for the origins of SIVmnd and other primate lentiviruses. *J Virol* **73**, 1036-1045.

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M. & Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**, 123-126.

Hofmann-Lehmann, R., Rasmussen, R. A., Vlasak, J., Smith, B. A., Baba, T. W., Liska, V., Montefiori, D. C., McClure, H. M., Anderson, D. C., Bernacky, B. J., Rizvi, T. A., Schmidt, R., Hill, L. R., Keeling, M. E., Katinger, H., Stiegler, G., Posner, M. R., Cavacini, L. A., Chou, T. C. & Ruprecht, R. M. (2001). Passive immunization against oral AIDS virus transmission: an approach to prevent mother-to-infant HIV-1 transmission? *J Med Primatol* **30**, 190-196.

Homann, D., Teyton, L. & Oldstone, M. B. (2001). Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat Med* **7**, 913-919.

Hori, T., Paliard, X., de Waal Malefijt, R., Ranes, M. & Spits, H. (1991). Comparative analysis of CD8 expressed on mature CD4+ CD8+ T cell clones cultured with IL-4 and that on CD8+ T cell clones: implication for functional significance of CD8 beta. *Int Immunol* **3**, 737-41.

Hsieh, S. M., Hung, C. C., Pan, S. C., Wang, J. T., Tsai, H. C., Chen, M. Y. & Chang, S. C. (2000). Restoration of cellular immunity against tuberculosis in patients coinfected with HIV-1 and tuberculosis with effective antiretroviral therapy: assessment by determination of CD69 expression on T cells after tuberculin stimulation. *J Acquir Immune Defic Syndr* **25**, 212-220.

Hu, W. S. & Temin, H. (1990). Genetic consequences of packaging two RNA genomes in one retroviral particle: pseudodiploidy and high rate of genetic recombination. *Proc Natl Acad Sci USA* **87**, 1556-1560.

Huard, B. & Karlsson, L. (2000). A subpopulation of CD8+ T cells specific for melanocyte differentiation antigens expresses killer inhibitory receptors (KIR) in healthy donors: evidence for a role of KIR in the control of peripheral tolerance. *Eur J Immunol* **30**, 1665-1675.

Hughes, E. S., Bell, J. E. & Simmonds, P. (1997a). Investigation of the dynamics of the spread of human immunodeficiency virus to brain and other tissues by evolutionary analysis of sequences from the p17(gag) and env genes. *J Virol* **71**, 1272-1280.

Hughes, E. S., Bell, J. E. & Simmonds, P. (1997b). Investigation of the dynamics of the spread of human immunodeficiency virus to brain and other tissues by evolutionary analysis of sequences from the p17gag and env genes. *J Virol* **71**, 1272-1280.

Hughes,G. (2005). Personal communication.

Imlach, S., Leen, C., Bell, J. E. & Simmonds, P. (2003). Phenotypic analysis of peripheral blood gammadelta T lymphocytes and their targeting by human immunodeficiency virus type 1 in vivo. *Virology* **305**, 415-427.

Imlach, S., McBreen, S., Shirafuji, T., Leen, C., Bell, J. E. & Simmonds, P. (2001). Activated peripheral CD8 lymphocytes express CD4 in vivo and are targets for infection by human immunodeficiency virus type 1. *J Virol* **75**, 11555-64.

Jansen, C. A., Piriou, E., Bronke, C., Vingerhoed, J., Kostense, S., Van Baarle, D. & Miedema, F. (2004). Characterization of virus-specific CD8(+) effector T cells in the course of HIV-1 infection: longitudinal analyses in slow and rapid progressors. *Clin Immunol* **113**, 299-309.

Jimenez, E., Sacedon, R., Vicente, A., Hernandez-Lopez, C., Zapata, A. G. & Varas, A. (2002). Rat peripheral CD4+CD8+ T lymphocytes are partially immunocompetent thymus-derived cells that undergo post-thymic maturation to become functionally mature CD4+ T lymphocytes. *J Immunol* **168**, 5005-5013.

Jin, X., Bauer, D. E., Tuttleton, S. E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C. E., Safrit, J. T., Mittler, J., Weinberger, L., Kostrikis, L. G., Zhang, L. Q., Perelson, A. S. & Ho, D. D. (1999). Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* **189**, 991-998.

Jin, X., Ramanathan, M., Jr., Barsoum, S., Deschenes, G. R., Ba, L., Binley, J., Schiller, D., Bauer, D. E., Chen, D. C., Hurley, A., Gebuhrer, L., El Habib, R., Caudrelier, P., Klein, M., Zhang, L., Ho, D. D. & Markowitz, M. (2002). Safety and immunogenicity of ALVAC vCP1452 and recombinant gp160 in newly human immunodeficiency virus type 1-infected patients treated with prolonged highly active antiretroviral therapy. *J Virol* **76**, 2206-2216.

Jobe, O., Ariyoshi, K., Marchant, A., Sabally, S., Corrah, T., Berry, N., Jaffar, S. & Whittle, H. (1999). Proviral load and immune function in blood and lymph node during HIV-1 and HIV-2 infection. *Clin Exp Immunol* **116**, 474-478.

Johnson, R. P. (2000). The dynamics of T-lymphocyte turnover in AIDS. *AIDS* **14 Suppl 3:S3-9.**, S3-S9.

Joshi, V. V., Oleske, J. M., Saad, S., Gadol, C., Connor, E., Bobila, R. & Minnefor, A. B. (1986). Thymus biopsy in children with acquired immunodeficiency syndrome. *Arch Pathol Lab Med* **110**, 837-842.

Kaech, S. M., Wherry, E. J. & Ahmed, R. (2002). Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol* **2**, 251-262.

Kahn, J. O. & Walker, B. D. (1998). Current concepts: Acute human immunodeficiency virus type 1 infection. *N Engl J Med* **339**, 33-39.

Kalams, S. A. & Walker, B. D. (1998). The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J Exp Med* **188**, 2199-2204.

Karlsson, A. C., Birk, M., Lindback, S., Gaines, H., Mittler, J. E. & Sonnerborg, A. (2001). Initiation of therapy during primary HIV type 1 infection results in a continuous decay of proviral DNA and a highly restricted viral evolution. *AIDS Res Hum Retroviruses* **17**, 409-416.

Kaslow, R. A., Carrington, M., Apple, R., Park, L., Munoz, A., Saah, A. J., Goedert, J. J., Winkler, C., O'Brien, S. J., Rinaldo, C., Detels, R., Blattner, W., Phair, J., Erlich, H. & Mann, D. L. (1996a). Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med* **2**, 405-411.

Kaslow, R. A., Carrington, M., Apple, R., Park, L., Munoz, A., Saah, A. J., Goedert, J. J., Winkler, C., Obrien, S. J., Rinaldo, C., Detels, R., Blattner, W., Phair, J., Erlich, H. & Mann, D. L. (1996b). Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nature Med* **2**, 405-411.

Kaur, A., Grant, R. M., Means, R. E., Mcclure, H., Feinberg, M. & Johnson, R. P. (1998). Diverse host responses and outcomes following simian immunodeficiency virus SIVmac239 infection in sooty mangabeys and rhesus macaques. *J Virol* **72**, 9597-9611.

Keane, N. M., Price, P., Lee, S., Almeida, C. A., Stone, S. F., James, I. & French, M. A. (2004). Restoration of CD4 T-cell responses to cytomegalovirus is short-lived in severely immunodeficient HIV-infected patients responding to highly active antiretroviral therapy. *HIV Med* **5**, 407-414.

Kedzierska, K., Azzam, R., Ellery, P., Mak, J., Jaworowski, A. & Crowe, S. M. (2003). Defective phagocytosis by human monocyte/macrophages following HIV-1 infection: underlying mechanisms and modulation by adjunctive cytokine therapy. *J Clin Virol* **26**, 247-263.

Kedzierska, K. & Crowe, S. M. (2001). Cytokines and HIV-1: interactions and clinical implications. *Antivir Chem Chemother* **12**, 133-150.

Kelleher, A. D., Carr, A., Zaunders, J. & Cooper, D. A. (1996). Alterations in the immune response of human immunodeficiency virus (HIV)-infected subjects treated with an HIV-specific protease inhibitor, ritonavir. *J Infect Dis* **173**, 321-329.

Kelleher, A. D., Long, C., Holmes, E. C., Allen, R. L., Wilson, J., Conlon, C., Workman, C., Shaunak, S., Olson, K., Goulder, P., Brander, C., Ogg, G., Sullivan, J. S., Dyer, W., Jones, I., McMichael, A. J., Rowland-Jones, S. & Phillips, R. E. (2001). Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. *J Exp Med* **193**, 375-386.

Kent, K. A., Kitchin, P., Mills, K. H. G., Page, M., Taffs, F., Corcoran, T., Silvera, P., Flanagan, B., Powell, C., Rose, J., Ling, C., Aubertin, A. M. & Stott, E. J. (1994). Passive immunization of cynomolgus macaques with immune sera or a pool of neutralizing monoclonal antibodies failed to protect against challenge with sivmac251. *AIDS Res Hum Retroviruses* **10**, 189-194.

Kern, F., Khatamzas, E., Surel, I., Frommel, C., Reinke, P., Waldrop, S. L., Picker, L. J. & Volk, H. D. (1999). Distribution of human CMV-specific memory T cells among the CD8pos. subsets defined by CD57, CD27, and CD45 isoforms. *Eur J Immunol* **29**, 2908-2915.

Kersten, M. J., Klein, M. R., Holwerda, A. M., Miedema, F. & vanOers, M. H. J. (1997). Epstein-Barr virus-specific cytotoxic T cell responses in HIV-1 infection - Different kinetics in patients progressing to opportunistic infection or non-Hodgkin's lymphoma. *J Clin Invest* **99**, 1525-1533.

Khatissian, E., Monceaux, V., Cumont, M. C., Ho Tsong, F. R., Estaquier, J. & Hurtrel, B. (2003). Simian immunodeficiency virus infection of CD4+CD8+ T cells in a macaque with an unusually high peripheral CD4+CD8+ T lymphocyte count. *AIDS Res Hum Retroviruses* **19**, 267-274.

Kirchhoff, F., Greenough, T. C., Brettler, D. B., Sullivan, J. L. & Desrosiers, R. C. (1995). Brief report: absence of intact nef sequences in a long- term survivor with nonprogressive HIV-1 infection. *N Engl J Med* **332** , 228-232.

Kitchen, S. G., Jones, N. R., LaForge, S., Whitmire, J. K., Vu, B. A., Galic, Z., Brooks, D. G., Brown, S. J., Kitchen, C. M. & Zack, J. A. (2004). CD4 on CD8(+) T cells directly enhances effector function and is a target for HIV infection. *Proc Natl Acad Sci U S A* **101**, 8727-8732.

Kitchen, S. G., Korin, Y. D., Roth, M. D., Landay, A. & Zack, J. A. (1998). Costimulation of naive CD8(+) lymphocytes induces CD4 expression and allows human immunodeficiency virus type 1 infection. *J Virol* **72**, 9054-9060.

Kitchen, S. G., LaForge, S., Patel, V. P., Kitchen, C. M., Miceli, M. C. & Zack, J. A. (2002). Activation of CD8 T cells induces expression of CD4, which functions as a chemotactic receptor. *Blood* **99**, 207-212.

Konig, R. & Zhou, W. (2004). Signal transduction in T helper cells: CD4 coreceptors exert complex regulatory effects on T cell activation and function. *Curr Issues Mol Biol* **6**, 1-15.

Kostense, S., Ogg, G. S., Manting, E. H., Gillespie, G., Joling, J., Vandenberghe, K., Veenhof, E. Z., Van Baarle, D., Jurriaans, S., Klein, M. R. & Miedema, F. (2001). High viral burden in the presence of major HIV-specific CD8(+) T cell expansions: evidence for impaired CTL effector function. *Eur J Immunol* **31**, 677-686.

Kostense, S., Otto, S. A., Knol, G. J., Manting, E. H., Nanlohy, N. M., Jansen, C., Lange, J. M., van Oers, M. H., Miedema, F. & Van Baarle, D. (2002a). Functional restoration of human immunodeficiency virus and Epstein-Barr virus-specific CD8(+) T cells during highly active antiretroviral therapy is associated with an increase in CD4(+) T cells. *Eur J Immunol* **32**, 1080-1089.

Kostense, S., Vandenberghe, K., Joling, J., Van Baarle, D., Nanlohy, N., Manting, E. & Miedema, F. (2002b). Persistent numbers of tetramer+ CD8(+) T cells, but loss of interferon-gamma+ HIV-specific T cells during progression to AIDS. *Blood* **99**, 2505-2511.

Koup, R. A., Safrit, J. T., Cao, Y. Z., Andrews, C. A., Mcleod, G., Borkowsky, W., Farthing, C. & Ho, D. D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* **68**, 4650-4655.

Kovacs, J. A., Lempicki, R. A., Sidorov, I. A., Adelsberger, J. W., Herpin, B., Metcalf, J. A., Sereti, I., Polis, M. A., Davey, R. T., Tavel, J., Falloon, J., Stevens, R., Lambert, L., Dewar, R., Schwartzentruber, D. J., Anver, M. R., Baseler, M. W., Masur, H., Dimitrov, D. S. & Lane, H. C. (2001). Identification of dynamically distinct subpopulations of T lymphocytes that are differentially affected by HIV. *J Exp Med* **194**, 1731-1741.

Kryworuchko, M., Pasquier, V., Keller, H., David, D., Goujard, C., Gilquin, J., Viard, J. P., Joussemet, M., Delfraissy, J. F. & Theze, J. (2004). Defective interleukin-2-dependent STAT5 signalling in CD8 T lymphocytes from HIV-positive patients: restoration by antiretroviral therapy. *AIDS* **18**, 421-426.

Kulkosky, J., Nunnari, G., Otero, M., Calarota, S., Dornadula, G., Zhang, H., Malin, A., Sullivan, J., Xu, Y., DeSimone, J., Babinchak, T., Stern, J., Cavert, W., Haase, A. & Pomerantz, R. J. (2002). Intensification and stimulation therapy for human immunodeficiency virus type 1 reservoirs in infected persons receiving virally suppressive highly active antiretroviral therapy. *J Infect Dis* **186**, 1403-1411.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J. & Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648-659.

Lafeuillade, A., Poggi, C., Chadapaud, S., Hittinger, G., Khiri, H. & Halfon, P. (2001). Impact of immune interventions on proviral HIV-1 DNA decay in patients receiving highly active antiretroviral therapy. *HIV Med* **2**, 189-194.

Lane, H. C., Masur, H., Edgar, L. C., Whalen, G., Rook, A. H. & Fauci, A. S. (1983). Abnormalities of B cell activation and immunoregulation in patients with acquired immunodeficiency syndrome. *N Engl J Med* **309**, 453-458.

Lascar, R. M., Lopes, A. R., Gilson, R. J., Dunn, C., Johnstone, R., Copas, A., Reignat, S., Webster, G., Bertoletti, A. & Maini, M. K. (2005). Effect of HIV infection and antiretroviral therapy on hepatitis B virus (HBV)-specific T cell responses in patients who have resolved HBV infection. *J Infect Dis* **191**, 1169-1179.

Laux, I., Khoshnan, A., Tindell, C., Bae, D., Zhu, X., June, C. H., Effros, R. B. & Nel, A. (2000). Response differences between human CD4(+) and CD8(+) T-cells during CD28 costimulation: implications for immune cell-based therapies and studies related to the expansion of double-positive T-cells during aging. *Clin Immunol* **96**, 187-197.

Lawn, S. D., Roberts, B. D., Griffin, G. E., Folks, T. M. & Butera, S. T. (2000). Cellular compartments of human immunodeficiency virus type 1 replication in vivo: Determination by presence of virion- associated host proteins and impact of opportunistic infection. *J Virol* **74**, 139-145.

Ledergerber, B., Mocroft, A., Reiss, P., Furrer, H., Kirk, O., Bickel, M., Uberti-Foppa, C., Pradier, C., d'Arminio, M. A., Schneider, M. M. & Lundgren, J. D. (2001). Discontinuation of secondary prophylaxis against Pneumocystis carinii pneumonia in patients with HIV infection who have a response to antiretroviral therapy. Eight European Study Groups. *N Engl J Med* **344**, 168-174.

Lee, S., Goldstein, H., Baseler, M., Adelsberger, J. & Golding, H. (1997). Human immunodeficiency virus type 1 infection of mature CD3(hi)CD8(+) thymocytes. *J Virol* **71**, 6671-6676.

Levacher, M., Hulstaert, F., Tallet, S., Ullery, S., Pocidalo, J. J. & Bach, B. A. (1992). The significance of activation markers on CD8 lymphocytes in human immunodeficiency syndrome: staging and prognostic value. *Clin Exp Immunol* **90**, 376-382.

Levy, J. A. (1998). Acute HIV infection and cells susceptible to HIV infection (Chapter 4), in: HIV and the pathogenesis of AIDS, 2nd Edition. Published by American Society for Microbiology.

Levy, J. A. (1993). HIV pathogenesis and long-term survival. *AIDS* **7**, 1401-1410.

Levy, J. A., Hoffman, A. D., Kramer, S. M., Landis, J. A. & Shimabukuro, J. M. (1984). Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* **225**, 840-842.

Levy, Y., Gahery-Segard, H., Durier, C., Lascaux, A. S., Goujard, C., Meiffredy, V., Rouzioux, C., Habib, R. E., Beumont-Mauviel, M., Guillet, J. G., Delfraissy, J. F. & Aboulker, J. P. (2005). Immunological and virological efficacy of a therapeutic immunization combined with interleukin-2 in chronically HIV-1 infected patients. *AIDS* **19**, 279-286.

Lewis, D. E., Tang, D. S. N., Aduoppong, A., Schober, W. & Rodgers, J. R. (1994). Anergy and apoptosis in CD8+ T cells from HIV-infected persons. *J Immunol* **153**, 412-420.

Li, Q., Duan, L., Estes, J. D., Ma, Z. M., Rourke, T., Wang, Y., Reilly, C., Carlis, J., Miller, C. J. & Haase, A. T. (2005). Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* **434**, 1148-1152.

Li, T. S., Tubiana, R., Katlama, C., Calvez, V., AitMohand, H. & Autran, B. (1998). Long-lasting recovery in CD4 T-cell function and viral-load reduction after highly active antiretroviral therapy in advanced HIV-1 disease. *Lancet* **351**, 1682-1686.

Lieberman, J., Fabry, J. A., Fong, D. M. & Parkerson, G. R. (1997). Recognition of a small number of diverse epitopes dominates the cytotoxic T lymphocyte response to HIV type 1 in an infected individual. *AIDS Res Hum Retroviruses* **13**, 383-392.

Lieberman, J., Shankar, P., Manjunath, N. & Andersson, J. (2001). Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection. *Blood* **98**, 1667-1677.

Lifson, A. R., Hilton, J. F., Westenhouse, J. L., Canchola, A. J., Samuel, M. C., Katz, M. H., Buchbinder, S. P., Hessol, N. A., Osmond, D. H., Shiboski, S., Lang, W., Greenspan, D. & Greenspan, J. S. (1994). Time from HIV seroconversion to oral candidiasis or hairy leukoplakia among homosexual and bisexual men enrolled in three prospective cohorts. *AIDS* **8**, 73-79.

Lifson, J. D., Feinberg, M., Reyes, G. R., Rabin, L., Banapour, B., Chakrabarti, S., Moss, B., Wong Staal, F., Steimer, K. & Engleman, E. (1986a). Induction of CD4 dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. *Nature* **323**, 725-728.

Lifson, J. D., Reyes, G. R., McGrath, M. S., Stein, B. S. & Engleman, E. G. (1986b). AIDS retrovirus induced cytopathology: giant cell formation and involvement of CD4 antigen. *Science* **232**, 1123-1127.

Lisziewicz, J., Bakare, N. & Lori, F. (2003). Therapeutic vaccination for future management of HIV/AIDS. *Vaccine* **21**, 620-623.

Lisziewicz, J., Rosenberg, E., Lieberman, J., Jessen, H., Lopalco, L., Siliciano, R., Walker, B. & Lori, F. (1999). Control of HIV despite the discontinuation of antiretroviral therapy. *N Engl J Med* **340**, 1683-1684.

Lisziewicz, J., Trocio, J., Whitman, L., Varga, G., Xu, J., Bakare, N., Erbacher, P., Fox, C., Woodward, R., Markham, P., Arya, S., Behr, J. P. & Lori, F. (2005). DermaVir: a novel topical vaccine for HIV/AIDS. *J Invest Dermatol* **124**, 160-169.

Liu, Z., Cumberland, W. G., Hultin, L. E., Prince, H. E., Detels, R. & Giorgi, J. V. (1997). Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV

disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. *J Acquir Immune Defic Syndr Hum Retrovirol* **16**, 83-92.

Livingstone, W. J., Moore, M., Innes, D., Bell, J. E., Simmonds, P., Whitelaw, J., Wyld, R., Robertson, J. R. & Brettle, R. P. (1996). Frequent infection of peripheral blood CD8-positive T-lymphocytes with HIV-1. *Lancet* **348**, 649-654.

Lopez-Cabrera, M., Santis, A. G., Fernandez-Ruiz, E., Blacher, R., Esch, F., Sanchez-Mateos, P. & Sanchez-Madrid, F. (1993). Molecular cloning, expression, and chromosomal localization of the human earliest lymphocyte activation antigen AIM/CD69, a new member of the C-type animal lectin superfamily of signal-transmitting receptors. *J Exp Med* **178**, 537-547.

Lori, F., Foli, A. & Lisziewicz, J. (2002). Structured treatment interruptions as a potential alternative therapeutic regimen for HIV-infected patients: a review of recent clinical data and future prospects. *J Antimicrob Chemother* **50**, 155-160.

Lori, F., Lewis, M. G., Xu, J., Varga, G., Zinn, D. E., Jr., Crabbs, C., Wagner, W., Greenhouse, J., Silvera, P., Yalley-Ogunro, J., Tinelli, C. & Lisziewicz, J. (2000). Control of SIV rebound through structured treatment interruptions during early infection. *Science* **290**, 1591-1593.

Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V. & Goff, S. P. (1993). Human immunodeficiency virus type-1 gag protein binds to cyclophilin-a and cyclophilin-B. *Cell* **73**, 1067-1078.

Lusso, P., De Maria, A., Malnati, M., Lori, F., DeRocco, S. E., Baseler, M. & Gallo, R. C. (1991). Induction of CD4 and susceptibility to HIV-1 infection in human CD8+ T lymphocytes by human herpesvirus 6. *Nature* **349**, 533-535.

Lusso, P., Lori, F. & Gallo, R. C. (1990). CD4-independent infection by human immunodeficiency virus type 1 after phenotypic mixing with human T-cell leukemia viruses. *J Virol* **64**, 6341-6344.

Lyles, R. H., Munoz, A., Yamashita, T. E., Bazmi, H., Detels, R., Rinaldo, C. R., Margolick, J. B., Phair, J. P. & Mellors, J. W. (2000). Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS Cohort Study. *J Infect Dis* **181**, 872-880.

Macatonia, S. E., Lau, R., Patterson, S., Pinching, A. J. & Knight, S. C. (1990). Dendritic cell infection, depletion and dysfunction in HIV-infected individuals. *Immunology* **71**, 38-45.

Macchi, B., Graziani, G., Zhang, J. & Mastino, A. (1993). Emergence of double-positive CD4/CD8 cells from adult peripheral blood mononuclear cells infected with human T cell leukemia virus type I (HTLV-I). *Cell Immunol* **149**, 376-389.

Macchia, D., Simonelli, C., Parronchi, P., Piccinni, M. P., Biswas, P., Mazzetti, M., Ravina, A., Maggi, E. & Romagnani, S. (1991). In vitro infection with HIV of antigen-specific T cell clones derived from HIV-seronegative individuals. Effects on cytokine production and helper function. *Ric Clin Lab* **21**, 85-90.

Macgregor, R. R., Boyer, J. D., Ugen, K. E., Lacy, K. E., Gluckman, S. J., Bagarazzi, M. L., Chattergoon, M. A., Baine, Y., Higgins, T. J., Ciccarelli, R. B., Coney, L. R., Ginsberg, R. S. & Weiner, D. B. (1998). First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: Safety and host response. *J Infect Dis* **178**, 92-100.

Mackall, C. L., Fleisher, T. A., Brown, M. R., Andrich, M. P., Chen, C. C., Feuerstein, I. M., Horowitz, M. E., Magrath, I. T., Shad, A. T., Steinberg, S. M., Wexler, L. H. & Gress, R. E. (1995). Age, thymopoiesis, and CD4+ t-lymphocyte regeneration after intensive chemotherapy. *N Engl J Med* **332**, 143-149.

Mackall, C. L., Fleisher, T. A., Brown, M. R., Andrich, M. P., Chen, C. C., Feuerstein, I. M., Magrath, I. T., Wexler, L. H., Dimitrov, D. S. & Gress, R. E. (1997). Distinctions between CD8+ and CD4+ T-cell regenerative pathways result in prolonged T-cell subset imbalance after intensive chemotherapy. *Blood* **89**, 3700-3707.

Maier, C. C., Bhandoola, A., Borden, W., Yui, K., Hayakawa, K. & Greene, M. I. (1998). Unique molecular surface features of in vivo tolerized T cells. *Proc Natl Acad Sci U S A* **95**, 4499-4503.

Mansky, L. M. (2000). In vivo analysis of human T-cell leukemia virus type 1 reverse transcription accuracy. *J Virol* **74**, 9525-9531.

Mansky, L. M. & Temin, H. M. (1995). Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J Virol* **69**, 5087-5094.

Marshall, J. D., Chehimi, J., Gri, G., Kostman, J. R., Montaner, L. J. & Trinchieri, G. (1999). The interleukin-12-mediated pathway of immune events is dysfunctional in human immunodeficiency virus-infected individuals. *Blood* **94**, 1003-1011.

Martinez-Marino, B., Shiboski, S., Hecht, F. M., Kahn, J. O. & Levy, J. A. (2004). Interleukin-2 therapy restores CD8 cell non-cytotoxic anti-HIV responses in primary infection subjects receiving HAART. *AIDS* **18**, 1991-1999.

Mascola, J. R., Stiegler, G., Vancott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., Beary, H., Hayes, D., Frankel, S. S., Birx, D. L. & Lewis, M. G. (2000). Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* **6**, 207-210.

Masur, H., Michelis, M. A., Greene, J. B., Onorato, I., Stouwe, R. A., Holzman, R. S., Wormser, G., Brettman, L., Lange, M., Murray, H. W. & Cunningham Rundles, S. (1981). An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. *N Engl J Med* **305**, 1431-1438.

Mathew, A., Kurane, I., Green, S., Vaughn, D. W., Kalayanarooj, S., Suntayakorn, S., Ennis, F. A. & Rothman, A. L. (1999). Impaired T cell proliferation in acute dengue infection. *J Immunol* **162**, 5609-5615.

Matloubian, M., Concepcion, R. J. & Ahmed, R. (1994). CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* **68**, 8056-8063.

Matsui, M., Fukuyama, H., Akiguchi, I. & Kameyama, M. (1989). Circulating CD4+CD8+ cells in myasthenia gravis: supplementary immunological parameter for long-term prognosis. *J Neurol* **236**, 329-335.

Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M. & Roederer, M. (2005). Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* **434**, 1093-1097.

McBreen, S., Imlach, S., Scott, G. R., Leen, C., Bell, J. E. & Simmonds, P. (2001). Preferential infection of the CD45RA+ (naive) subset of CD8+ lymphocytes by human immunodeficiency virus type 1 in vivo. *J Virol* **75**, 4091-4102.

McCune, J. M., Hanley, M. B., Cesar, D., Halvorsen, R., Hoh, R., Schmidt, D., Wieder, E., Deeks, S., Siler, S., Neese, R. & Hellerstein, M. (2000). Factors influencing T-cell turnover in HIV-1-seropositive patients [see comments]. *J Clin Invest 2000 Mar;105(5):R1-8* **105**, R1-8.

McElrath, M. J., Steinman, R. M. & Cohn, Z. A. (1991). Latent HIV-1 infection in enriched populations of blood monocytes and T cells from seropositive patients. *J Clin Invest* **87**, 27-30.

McFarland, R. D., Douek, D. C., Koup, R. A. & Picker, L. J. (2000). Identification of a human recent thymic emigrant phenotype. *Proc Natl Acad Sci U S A* **97**, 4215-4220.

McGhee, J. D. & von Hippel, P. H. (1977). Formaldehyde as a probe of DNA structure. 3. Equilibrium denaturation of DNA and synthetic polynucleotides. *Biochemistry* **16**, 3267-3276.

McKay, P. F., Barouch, D. H., Schmitz, J. E., Veazey, R. S., Gorgone, D. A., Lifton, M. A., Williams, K. C. & Letvin, N. L. (2003). Global dysfunction of CD4 T-lymphocyte cytokine expression in simian-human immunodeficiency virus/SIV-infected monkeys is prevented by vaccination. *J Virol* **77**, 4695-4702.

McMichael, A. J. & Rowland-Jones, S. L. (2001). Cellular immune responses to HIV. *Nature* **%19;410**, 980-987.

McNeil, A. C., Shupert, W. L., Iyasere, C. A., Hallahan, C. W., Mican, J. A., Davey, R. T., Jr. & Connors, M. (2001). High-level HIV-1 viremia suppresses viral antigen-specific CD4(+) T cell proliferation. *Proc Natl Acad Sci U S A* **98**, 13878-13883.

Mehandru, S., Poles, M. A., Tenner-Racz, K., Horowitz, A., Hurley, A., Hogan, C., Boden, D., Racz, P. & Markowitz, M. (2004). Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* **200**, 761-770.

Meireles-de-Souza, L. R. & Shattock, R. J. (2005). Therapeutic role of CD8+ T cells in HIV-1 infection: targets and suppressors of viral replication. *Expert Opin Biol Ther* **5**, 321-332.

Mercure, L., Brenner, B. J., Phaneuf, D., Tsoukas, C. & Wainberg, M. A. (1994). Effect of 3'-azido-3'-deoxythymidine and 2',3'-dideoxyinosine on establishment of human immunodeficiency virus type 1 infection in cultured CD8+ lymphocytes. *Antimicrob Agents Chemother* **38**, 986-990.

Merrill, J. E., Koyanagi, Y. & Chen, I. S. (1989). Interleukin-1 and tumor necrosis factor alpha can be induced from mononuclear phagocytes by human immunodeficiency virus type 1 binding to the CD4 receptor. *J Virol* **63**, 4404-4408.

Meyaard, L., Otto, S. A., Hooibrink, B. & Miedema, F. (1994a). Quantitative analysis of CD4(+) t cell function in the course of human immunodeficiency virus infection - gradual decline of both naive and memory alloreactive t cells. *J Clin Invest* **94**, 1947-1952.

Meyaard, L., Otto, S. A., Jonker, R. R., Mijnster, M. J., Keet, R. P. & Miedema, F. (1992). Programmed death of T cells in HIV-1 infection. *Science* **257**, 217-219.

Meyaard, L., Otto, S. A., Keet, I. P. M., Vanlier, R. A. W. & Miedema, F. (1994b). Changes in cytokine secretion patterns of CD4(+) t-cell clones in human immunodeficiency virus infection. *Blood* **84**, 4262-4268.

Michael, N. L. & Burke, D. S. (1991). Natural history of human immunodeficiency virus infection. *Dermatol Clin* **9**, 429-441.

Miedema, F., Petit, A. J., Terpstra, F. G., Schattenkerk, J. K., de Wolf, F., Al, B. J. M., Roos, M., Lange, J. M., Danner, S. A. & Goudsmit, J. (1988). Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men. HIV affects the immune system before CD4+ T helper cell depletion occurs. *J Clin Invest* **82**, 1908-1914.

Migueles, S. A. & Connors, M. (2001). Frequency and function of HIV-specific CD8(+) T cells. *Immunol Lett* **79**, 141-150.

Migueles, S. A., Laborico, A. C., Shupert, W. L., Sabbaghian, M. S., Rabin, R., Hallahan, C. W., Van Baarle, D., Kostense, S., Miedema, F., McLaughlin, M., Ehler, L., Metcalf, J., Liu, S. & Connors, M. (2002). HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* **3**, 1061-1068.

Mikhail, M., Wang, B. & Saksena, N. K. (2003). Mechanisms involved in non-progressive HIV disease. *AIDS Rev* **5**, 230-244.

Miller, M. A., Cloyd, M. W., Liebmann, J., Rinaldo, C. R., Islam, K. R., Wang, S. Z. S., Mietzner, T. A. & Montelaro, R. C. (1993). Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. *Virology* **196**, 89-100.

Mizuki, M., Tagawa, S., Machii, T., Shibano, M., Tatsumi, E., Tsubaki, K., Tako, H., Yokohama, A., Satou, S., Nojima, J., Hirota, T. & Kitani, T. (1998). Phenotypical heterogeneity of CD4+CD8+ double-positive chronic T lymphoid leukemia. *Leukemia* **12**, 499-504.

Mohri, H., Bonhoeffer, S., Monard, S., Perelson, A. S. & Ho, D. D. (1998). Rapid turnover of T lymphocytes in SIV-infected rhesus macaques. *Science* **279**, 1223-1227.

Mohri, H., Perelson, A. S., Tung, K., Ribeiro, R. M., Ramratnam, B., Markowitz, M., Kost, R., Hurley, A., Weinberger, L., Cesar, D., Hellerstein, M. K. & Ho, D. D. (2001). Increased turnover of T lymphocytes in HIV-1 infection and its reduction by antiretroviral therapy. *J Exp Med* **194**, 1277-1287.

Moore, J. P., Parren, P. W. & Burton, D. R. (2001). Genetic subtypes, humoral immunity, and human immunodeficiency virus type 1 vaccine development. *J Virol* **75**, 5721-5729.

Moskophidis, D., Lechner, F., Pircher, H. & Zinkernagel, R. M. (1993). Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* **362**, 758-761.

Moss, P. A. H., Rowlandjones, S. L., Frodsham, P. M., Mcadam, S., Giangrande, P., McMichael, A. J. & Bell, J. I. (1995). Persistent high frequency of human immunodeficiency virus- specific cytotoxic t cells in peripheral blood of infected donors. *Proc Natl Acad Sci USA* **92**, 5773-5777.

Mueller, Y. M., De Rosa, S. C., Hutton, J. A., Witek, J., Roederer, M., Altman, J. D. & Katsikis, P. D. (2001) . Increased CD95/Fas-induced apoptosis of HIV-specific CD8(+) T cells. *Immunity* **15**, 871-882.

Murray, H. W., Scavuzzo, D. A., Kelly, C. D., Rubin, B. Y. & Roberts, R. B. (1988). T4+ cell production of interferon gamma and the clinical spectrum of patients at risk for and with acquired immunodeficiency syndrome. *Arch Intern Med* **148**, 1613-1616.

Musey, L., Hughes, J., Schacker, T., Shea, T., Corey, L. & McElrath, M. J. (1997). Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. *N Engl J Med* **337**, 1267-1274.

Musey, L. K., Krieger, J. N., Hughes, J. P., Schacker, T., Corey, L. & McElrath, M. J. (1999). Early and persistent human immunodeficiency virus type 1 (HIV-l)- specific T helper dysfunction in blood and lymph nodes following acute HIV-1 infection. *J Infec Dis* **180**, 278-284.

Nam, K., Akari, H., Terao, K., Shibata, H., Kawamura, S. & Yoshikawa, Y. (2000). Peripheral blood extrathymic CD4(+)CD8(+) T cells with high cytotoxic activity are from the same lineage as CD4(+)CD8(-) T cells in cynomolgus monkeys. *Int Immunol* **12**, 1095-1103.

Nascimbeni, M., Shin, E. C., Chiriboga, L., Kleiner, D. E. & Rehermann, B. (2004). Peripheral CD4(+)CD8(+) T cells are differentiated effector memory cells with antiviral functions. *Blood* **104**, 478-486.

Nicholson, J. K., Rao, P. E., Calvelli, T., Stetler-Stevenson, M., Browning, S. W., Yeung, L. & Marti, G. E. (1994). Artifactual staining of monoclonal antibodies in two-color combinations is due to an immunoglobulin in the serum and plasma. *Cytometry* **18**, 140-146.

Noguchi, M., Furuya, S., Takeuchi, T. & Hirohashi, S. (1997). Modified formalin and methanol fixation methods for molecular biological and morphological analyses. *Pathol Int* **47**, 685-691.

Norazmi, M. N., Arifin, H. & Jamaruddin, M. A. (1995). Increased level of activated gamma delta lymphocytes correlates with disease severity in HIV infection. *Immunol Cell Biol* **73**, 245-248.

Norment, A. M. & Littman, D. R. (1988). A second subunit of CD8 is expressed in human T cells. *EMBO J* **7**, 3433-3439.

Ober, B. T., Summerfield, A., Mattlinger, C., Wiesmuller, K. H., Jung, G., Pfaff, E., Saalmuller, A. & Rziha, H. J. (1998). Vaccine-induced, pseudorabies virus-specific, extrathymic CD4+CD8+ memory T-helper cells in swine. *J Virol* **72**, 4866-4873.

Ogg, G. S., Jin, X., Bonhoeffer, S., Dunbar, P. R., Nowak, M. A., Monard, S., Segal, J. P., Cao, Y. Z., Rowlandjones, S. L., Cerundolo, V., Hurley, A., Markowitz, M., Ho, D. D., Nixon, D. F. & McMichael, A. J. (1998). Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* **279**, 2103-2106.

Ogg, G. S., Kostense, S., Klein, M. R., Jurriaans, S., Hamann, D., McMichael, A. J. & Miedema, F. (1999). Longitudinal phenotypic analysis of human immunodeficiency virus type 1-specific cytotoxic T lymphocytes: Correlation with disease progression. *J Virol* **73**, 9153-9160.

Okada, H., Takei, R. & Tashiro, M. (1998). Inhibition of HIV-1 Nef-induced apoptosis of uninfected human blood cells by serine/threonine protein kinase inhibitors, fasudil hydrochloride and M3. *FEBS Lett* **422**, 363-367.

Orenstein, J. M., Meltzer, M. S., Phipps, T. & Gendelman, H. E. (1988). Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor-1-treated human monocytes: an ultrastructural study. *J Virol* **62**, 2578-2586.

Ortolani, C., Forti, E., Radin, E., Cibin, R. & Cossarizza, A. (1993). Cytofluorimetric identification of two populations of double positive (CD4+,CD8+) T lymphocytes in human peripheral blood. *Biochem Biophys Res Commun* **191**, 601-609.

Oyaizu, N., Chirmule, N., Kalyanaraman, V. S., Hall, W. W., Pahwa, R., Shuster, M. & Pahwa, S. (1990). Human immunodeficiency virus type 1 envelope glycoprotein gp120 produces immune defects in CD4+ T lymphocytes by inhibiting interleukin 2 mRNA. *Proc Natl Acad Sci U S A* **87**, 2379-2383.

Pantaleo, G., De Maria, A., Koenig, S., Butini, L., Moss, B., Baseler, M., Lane, H. C. & Fauci, A. S. (1990). CD8+ T lymphocytes of patients with AIDS maintain normal broad cytolytic function despite the loss of human immunodeficiency virus-specific cytotoxicity. *Proc Natl Acad Sci U S A* **87**, 4818-4822.

Pantaleo, G., Demarest, J. F., Schacker, T., Vaccarezza, M., Cohen, O. J., Daucher, M., Graziosi, C., Schnittman, S. S., Quinn, T. C., Shaw, G. M., Perrin, L., Tambussi, G., Lazzarin, A., Sekaly, R. P., Soudeyns, H., Corey, L. & Fauci, A. S. (1997). The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia. *Proc Natl Acad Sci USA* **94**, 254-258.

Pantaleo, G., Graziosi, C., Butini, L., Pizzo, P. A., Schnittman, S. M., Kotler, D. P. & Fauci, A. S. (1991). Lymphoid organs function as major reservoirs for human immunodeficiency virus. *Proc Natl Sci USA* **88**, 9838-9842.

Pantaleo, G., Graziosi, C., Demarest, J. F., Butini, L., Montroni, M., Fox, C. H., Orenstein, J. M., Kotler, D. P. & Fauci, A. S. (1993). HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* **362**, 355-358.

Parker, N. G., Notermans, D. W., Deboer, R. J., Roos, M. T. L., Dewolf, F., Hill, A., Leonard, J. M., Danner, S. A., Miedema, F. & Schellekens, P. T. A. (1998). Biphasic kinetics of peripheral blood T cells after triple combination therapy in HIV-1 infection: A composite of redistribution and proliferation. *Nature Med* **4**, 208-214.

Peakman, M., Mahalingam, M., Pozniak, A., Mcmanus, T. J., Phillips, A. N. & Vergani, D. (1995). Markers of immune cell activation and disease progression. Cell activation in HIV disease. *Adv Exp Med Biol* **374:17-26.**, 17-26.

Perelson, A. S., Essunger, P., Cao, Y. Z., Vesanen, M., Hurley, A., Saksela, K., Markowitz, M. & Ho, D. D. (1997). Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature* **387**, 188-191.

Periwal, S. B. & Cebra, J. J. (1999). Respiratory mucosal immunization with reovirus serotype 1/L stimulates virus-specific humoral and cellular immune responses, including double-positive (CD4(+)/CD8(+)) T cells. *J Virol* **73**, 7633-7640.

Petrovas, C., Mueller, Y. M. & Katsikis, P. D. (2004). HIV-specific CD8+ T cells: serial killers condemned to die? *Curr HIV Res* **2**, 153-162.

Picker, L. J., Hagen, S. I., Lum, R., Reed-Inderbitzin, E. F., Daly, L. M., Sylwester, A. W., Walker, J. M., Siess, D. C., Piatak, M., Jr., Wang, C., Allison, D. B., Maino, V. C., Lifson, J. D., Kodama, T. & Axthelm, M. K. (2004). Insufficient production and tissue delivery of CD4+ memory T cells in rapidly progressive simian immunodeficiency virus infection. *J Exp Med* **200** , 1299-1314.

Pierson, T., Mcarthur, J. & Siliciano, R. F. (2000). Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. *Annu Rev Immunol* **18:665-708.**, 665-708.

Piguet, V. & Trono, D. (2001). Living in oblivion: HIV immune evasion. *Semin Immunol* **13**, 51-57.

Pillay, D. (2004). Current patterns in the epidemiology of primary HIV drug resistance in North America and Europe. *Antivir Ther* **9**, 695-702.

Pitcher, C. J., Quittner, C., Peterson, D. M., Connors, M., Koup, R. A., Maino, V. C. & Picker, L. J. (1999). HIV-1-specific CD4(+) T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nature Med* **5**, 518-525.

Potempa, S., Picard, L., Reeves, J. D., Wilkinson, D., Weiss, R. A. & Talbot, S. J. (1997). CD4-independent infection by human immunodeficiency virus type 2 strain ROD/B: the role of the N-terminal domain of CXCR-4 in fusion and entry. *J Virol* **71**, 4419-4424.

Potter, S. J., Dwyer, D. E. & Saksena, N. K. (2003). Differential cellular distribution of HIV-1 drug resistance in vivo: evidence for infection of CD8+ T cells during HAART. *Virology* **305**, 339-352.

Prince, H. E., Golding, J. & York, J. (1994). Characterization of circulating CD4+ CD8+ lymphocytes in healthy individuals prompted by identification of a blood donor with a markedly elevated level of CD4+ CD8+ lymphocytes. *Clin Diagn Lab Immunol* **1**, 597-605.

Quintana, F. J., Gerber, D., Kent, S. C., Cohen, I. R. & Shai, Y. (2005). HIV-1 fusion peptide targets the TCR and inhibits antigen-specific T cell activation. *J Clin Invest* **115**, 2149-2158.

Reeves, J. D. & Doms, R. W. (2002). Human immunodeficiency virus type 2. *J Gen Virol* **83**, 1253-1265.

Reeves, J. D. & Schulz, T. F. (1997). The CD4-independent tropism of human immunodeficiency virus type 2 involves several regions of the envelope protein and correlates with a reduced activation threshold for envelope-mediated fusion. *J Virol* **71**, 1453-1465.

Reimann, K. A., Tennerracz, K., Racz, P., Montefiori, D. C., Yasutomi, Y., Lin, W. Y., Ransil, B. J. & Letvin, N. L. (1994). Immunopathogenic events in acute infection of rhesus monkeys with simian immunodeficiency virus of macaques. *J Virol* **68**, 2362-2370.

Reitter, J. N., Means, R. E. & Desrosiers, R. C. (1998). A role for carbohydrates in immune evasion in AIDS. *Nature Med* **4**, 679-684.

ROBBINS, J. H. (1964). TISSUE CULTURE STUDIES OF THE HUMAN LYMPHOCYTE. *Science* **146:1648-54.**, 1648-1654.

Rodrigo, A. G., Goracke, P. C., Rowhanian, K. & Mullins, J. I. (1997). Quantitation of target molecules from polymerase chain reaction- based limiting dilution assays. *AIDS Res Hum Retroviruses* **13**, 737-742.

Roederer, M., Dubs, J. G., Anderson, M. T., Raju, P. A. & Herzenberg, L. A. (1995). CD8 naive T cell counts decrease progressively in HIV- infected adults. *J Clin Invest* **95**, 2061-2066.

Rook, A. H., Masur, H., Lane, H. C., Frederick, W., Kasahara, T., Macher, A. M., Djeu, J. Y., Manischewitz, J. F., Jackson, L., Fauci, A. S. & Quinnan, G. V., Jr. (1983). Interleukin-2 enhances the depressed natural killer and cytomegalovirus-specific cytotoxic activities of lymphocytes from patients with the acquired immune deficiency syndrome. *J Clin Invest* **72**, 398-403.

Roos, M. T., van Lier, R. A., Hamann, D., Knol, G. J., Verhoofstad, I., Van Baarle, D., Miedema, F. & Schellekens, P. T. (2000). Changes in the composition of circulating CD8+ T cell subsets during acute epstein-barr and human immunodeficiency virus infections in humans. *J Infect Dis* **182**, 451-458.

Rosenberg, E. S., Altfeld, M., Poon, S. H., Phillips, M. N., Wilkes, B. M., Eldridge, R. L., Robbins, G. K., D'Aquila, R. T., Goulder, P. J. & Walker, B. D. (2000). Immune control of HIV-1 after early treatment of acute infection. *Nature* **407**, 523-526.

Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A. & Walker, B. D. (1997). Vigorous HIV-1-specific CD4(+) T cell responses associated with control of viremia. *Science* **278**, 1447-1450.

Rosenberg, E. S., LaRosa, L., Flynn, T., Robbins, G. & Walker, B. D. (1999). Characterization of HIV-1-specific T-helper cells in acute and chronic infection. *Immunol Lett* **66**, 89-93.

Rosenstein, Y., Burakoff, S. J. & Herrmann, S. H. (1990). HIV-gp120 can block CD4-class II MHC-mediated adhesion. *J Immunol* **144**, 526-531.

Rubbert, A., Wohrmann, A., Passon, D., Jütte, A., Salzberger, B. & Fätkenheuer, G. (2005). Course of HIV RNA in CD4+ and CD8+ lymphocytes in HIV infected patients undergoing HAART as determined by ultrasensitive fluorescence *in situ* hybridisation (UFISH). **9th conference on retroviruses and opportunistic infections, poster 407-T**.

Sachsenberg, N., Perelson, A. S., Yerly, S., Schockmel, G. A., Leduc, D., Hirschel, B. & Perrin, L. (1998). Turnover of CD4(+) and CD8(+) T lymphocytes in HIV-1 infection as measured by Ki-67 antigen. *J Exp Med* **187**, 1295-1303.

Saha, K., Zhang, J. & Zerhouni, B. (2001). Evidence of productively infected CD8+ T cells in patients with AIDS: implications for HIV-1 pathogenesis. *J Acquir Immune Defic Syndr* **26**, 199-207.

Sala, P., Tonutti, E., Feruglio, C., Florian, F. & Colombatti, A. (1993). Persistent expansions of CD4+ CD8+ peripheral blood T cells. *Blood* **82**, 1546-1552.

Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708-712.

Scarlatti, G., Tresoldi, E., Bjorndal, A., Fredriksson, R., Colognesi, C., Deng, H. K., Malnati, M. S., Plebani, A., Siccardi, A. G., Littman, D. R., Fenyo, E. M. & Lusso, P. (1997). In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. *Nature Med* **3**, 1259-1265.

Schacker, T. W., Nguyen, P. L., Beilman, G. J., Wolinsky, S., Larson, M., Reilly, C. & Haase, A. T. (2002). Collagen deposition in HIV-1 infected lymphatic tissues and T cell homeostasis. *J Clin Invest* **110**, 1133-1139.

Schechter, M., Harrison, L. H., Halsey, N. A., Trade, G., Santino, M., Moulton, L. H. & Quinn, T. C. (1994). Coinfection with human t-cell lymphotropic virus type-i and HIV in brazil - impact on markers of HIV disease progression. *JAMA* **271**, 353-357.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tennerracz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L. & Reimann, K. A. (1999). Control of viremia in simian immunodeficiency virus infection by CD8(+) lymphocytes. *Science* **283**, 857-860.

Schnittman, S. M., Lane, H. C., Greenhouse, J., Justement, J. S., Baseler, M. & Fauci, A. S. (1990). Preferential infection of CD4+ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals. *Proc Natl Acad Sci U S A* **87**, 6058-6062.

Schnittman, S. M., Lane, H. C., Higgins, S. E., Folks, T. & Fauci, A. S. (1986). Direct polyclonal activation of human B lymphocytes by the acquired immune deficiency syndrome virus. *Science* **233**, 1084-1086.

Schwartz, O., Marechal, V., Le Gall, S., Lemonnier, F. & Heard, J. M. (1996). Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat Med* **2**, 338-342.

Scott, C. S., Wheeler, R., Ford, P., Bynoe, A. G. & Roberts, B. E. (1983). T lymphocyte subpopulations in idiopathic thrombocytopenic purpura (ITP). *Scand J Haematol* **30**, 401-406.

Semenzato, G., Agostini, C., Chieco-Bianchi, L. & De Rossi, A. (1998). HIV load in highly purified CD8+ T cells retrieved from pulmonary and blood compartments. *J Leukoc Biol* **64**, 298-301.

Semenzato, G., Agostini, C., Ometto, L., Zambello, R., Trentin, L., Chiecobianchi, L. & Derossi, A. (1995). CD8(+) t lymphocytes in the lung of acquired immunodeficiency syndrome patients harbor human immunodeficiency virus type 1. *Blood* **85**, 2308-2314.

Sereti, I., Anthony, K. B., Martinez-Wilson, H., Lempicki, R., Adelsberger, J., Metcalf, J. A., Hallahan, C. W., Follmann, D., Davey, R. T., Kovacs, J. A. & Lane, H. C. (2004). IL-2-induced CD4+ T-cell expansion in HIV-infected patients is associated with long-term decreases in T-cell proliferation. *Blood* **104**, 775-780.

Seth, A., Ourmanov, I., Schmitz, J. E., Kuroda, M. J., Lifton, M. A., Nickerson, C. E., Wyatt, L., Carroll, M., Moss, B., Venzon, D., Letvin, N. L. & Hirsch, V. M. (2000). Immunization with a modified vaccinia virus expressing simian immunodeficiency virus (SIV) Gag-Pol primes for an anamnestic Gag-specific cytotoxic T-lymphocyte response and is associated with reduction of viremia after SIV challenge. *J Virol* **74**, 2502-2509.

Shankar, P., Russo, M., Harnisch, B., Patterson, M., Skolnik, P. & Lieberman, J. (2000). Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. *Blood* **96**, 3094-3101.

Sharma, B. & Gupta, S. (1985). Antigen-specific primary cytotoxic T lymphocyte (CTL) responses in acquired immune deficiency syndrome (AIDS) and AIDS-related complexes (ARC). *Clin Exp Immunol* **62**, 296-303.

Shaw, G. M., Hahn, B. H., Arya, S. K., Groopman, J. E., Gallo, R. C. & Wong Staal, F. (1984). Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. *Science* **226**, 1165-1171.

Shearer, G. M., Bernstein, D. C., Tung, K. S., Via, C. S., Redfield, R., Salahuddin, S. Z. & Gallo, R. C. (1986). A model for the selective loss of major histocompatibility complex self-restricted T cell immune responses during the development of acquired immune deficiency syndrome (AIDS). *J Immunol* **137**, 2514-2521.

Shearer, G. M. & Clerici, M. (1991). Early T-helper cell defects in HIV infection. *AIDS* **5**, 245-253.

Shiue, L., Gorman, S. D. & Parnes, J. R. (1988). A second chain of human CD8 is expressed on peripheral blood lymphocytes. *J Exp Med* **168**, 1993-2005.

Sieg, S. F., Bazdar, D. A., Harding, C. V. & Lederman, M. M. (2001). Differential expression of interleukin-2 and gamma interferon in human immunodeficiency virus disease. *J Virol* **75**, 9983-9985.

Simmonds, P., Balfe, P., Peutherer, J. F., Ludlam, C. A., Bishop, J. O. & Leigh Brown, A. J. (1990). Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. *J Virol* **64**, 864-872.

Simmonds, P., Beatson, D., Cuthbert, R. J. G., Watson, H. G., Reynolds, B., Peutherer, J. F., Parry, J. V., Ludlam, C. A. & Steel, C. M. (1991). Determinants of HIV disease progression: a 6-year longitudinal study in the Edinburgh haemophilia/HIV cohort. *Lancet* **338**, 1159-1163.

Simon, F., Mauclere, P., Roques, P., Loussertajaka, I., MullerTrutwin, M. C., Saragosti, S., Georgescourbot, M. C., Barresinoussi, F. & Brunvezinet, F. (1998). Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nature Med* **4**, 1032-1037.

Sousa, A. E., Carneiro, J., Meier-Schellersheim, M., Grossman, Z. & Victorino, R. M. (2002). CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. *J Immunol* **169**, 3400-3406.

Speck, R. F., Esser, U., Penn, M. L., Eckstein, D. A., Pulliam, L., Chan, S. Y. & Goldsmith, M. A. (1999). A trans-receptor mechanism for infection of CD4-negative cells by human immunodeficiency virus type 1. *Curr Biol* **9**, 547-550.

Stanley, S. K., McCune, J. M., Kaneshima, H., Justement, J. S., Sullivan, M., Boone, E., Baseler, M., Adelsberger, J., Bonyhadi, M., Orenstein, J., Fox, C. H. & Fauci, A. S. (1993). Human immunodeficiency virus infection of the human thymus and disruption of the thymic microenvironment in the SCID- hu mouse. *J Exp Med* **178**, 1151-1163.

Stevenson, M. (2003). HIV-1 pathogenesis. *Nat Med* **9**, 853-860.

Stove, V., Van, d. W., I, Naessens, E., Coene, E., Stove, C., Plum, J. & Verhasselt, B. (2005). Human immunodeficiency virus Nef induces rapid internalization of the T-cell coreceptor CD8alphabeta. *J Virol* **79**, 11422-11433.

Su, L., Kaneshima, l., Bonyhadi, M., Salimi, S., Kraft, D., Rabin, L. & McCue, J. M. (1995). HIV induced thymocyte depletion is associated with indirect cytopathicity and infection of progenitor cells in vivo. *Immunity* **2**, 25.

Sullivan, Y. B., Landay, A. L., Zack, J. A., Kitchen, S. G. & Al Harthi, L. (2001). Upregulation of CD4 on CD8+ T cells: CD4dimCD8bright T cells constitute an activated phenotype of CD8+ T cells. *Immunology* **103**, 270-280.

Suni, M. A., Ghanekar, S. A., Houck, D. W., Maecker, H. T., Wormsley, S. B., Picker, L. J., Moss, R. B. & Maino, V. C. (2001). CD4(+)CD8(dim) T lymphocytes exhibit enhanced cytokine expression, proliferation and cytotoxic activity in response to HCMV and HIV-1 antigens. *Eur J Immunol* **31**, 2512-2520.

Swingler, S., Brichacek, B., Jacque, J. M., Ulich, C., Zhou, J. & Stevenson, M. (2003). HIV-1 Nef intersects the macrophage CD40L signalling pathway to promote resting-cell infection. *Nature* **424**, 213-219.

Takahashi, K., Wesselingh, S. L., Griffin, D. E., Mcarthur, J. C., Johnson, R. T. & Glass, J. D. (1996). Localization of HIV-1 in human brain using polymerase chain reaction/in situ hybridization and immunocytochemistry. *Ann Neurol* **39**, 705-711.

Tas, M., Drexhage, H. A. & Goudsmit, J. (1988). A monocyte chemotaxis inhibiting factor in serum of HIV infected men shares epitopes with the HIV transmembrane protein gp41. *Clin Exp Immunol* **71**, 13-18.

Tateno, M. & Levy, J. A. (1988). MT-4 plaque formation can distinguish cytopathic subtypes of the human immunodeficiency virus (HIV). *Virology* **167**, 299-301.

Teague, T. K., Munn, L., Zygourakis, K. & McIntyre, B. W. (1993). Analysis of lymphocyte activation and proliferation by video microscopy and digital imaging. *Cytometry* **14**, 772-782.

Teixeira, L., Valdez, H., McCune, J. M., Koup, R. A., Badley, A. D., Hellerstein, M. K., Napolitano, L. A., Douek, D. C., Mbisa, G., Deeks, S., Harris, J. M., Barbour, J. D., Gross, B. H., Francis, I. R., Halvorsen, R., Asaad, R. & Lederman, M. M. (2001). Poor CD4 T cell restoration after suppression of HIV-1 replication may reflect lower thymic function. *AIDS* **15**, 1749-1756.

Tokuda, Y., Nakamura, T., Satonaka, K., Maeda, S., Doi, K., Baba, S. & Sugiyama, T. (1990). Fundamental study on the mechanism of DNA degradation in tissues fixed in formaldehyde. *J Clin Pathol* **43**, 748-751.

Tonutti, E., Sala, P., Feruglio, C., Yin, Z. & Colombatti, A. (1994). Phenotypic Heterogeneity of Persistent Expansions of CD4+CD8+ T Cells. *Clinical Immunology and Immunopathology* **73**, 312-320.

Touloumi, G. & Hatzakis, A. (2000). Natural history of HIV-1 infection. *Clin Dermatol* **18**, 389-399.

Trepel, F. (1974). Number and distribution of lymphocytes in man. A critical analysis. *Klin Wochenschr* **52**, 511-515.

Trimble, L. A. & Lieberman, J. (1998). Circulating CD8 T lymphocytes in human immunodeficiency virus- infected individuals have impaired function and downmodulate CD3 zeta, the signaling chain of the T-cell receptor complex. *Blood* **91**, 585-594.

Ulmer, J. B., Fu, T. M., Deck, R. R., Friedman, A., Guan, L., DeWitt, C., Liu, X., Wang, S., Liu, M. A., Donnelly, J. J. & Caulfield, M. J. (1998). Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. *J Virol* **72**, 5648-5653.

Van Baarle, D., Hovenkamp, E., Callan, M. F., Wolthers, K. C., Kostense, S., Tan, L. C., Niesters, H. G., Osterhaus, A. D., McMichael, A. J., van Oers, M. H. & Miedema, F. (2001). Dysfunctional Epstein-Barr virus (EBV)-specific CD8(+) T lymphocytes and increased EBV load in HIV-1 infected individuals progressing to AIDS-related non-Hodgkin lymphoma. *Blood* **98**, 146-155.

Van Baarle, D., Kostense, S., Hovenkamp, E., Ogg, G., Nanlohy, N., Callan, M. F., Dukers, N. H., McMichael, A. J., van Oers, M. H. & Miedema, F. (2002a). Lack of Epstein-Barr virus- and HIV-specific CD27- CD8+ T cells is associated with progression to viral disease in HIV-infection. *AIDS* **16**, 2001-2011.

Van Baarle, D., Kostense, S., van Oers, M. H., Hamann, D. & Miedema, F. (2002b). Failing immune control as a result of impaired CD8+ T-cell maturation: CD27 might provide a clue. *Trends Immunol* **23**, 586-591.

Van Baarle, D., Wolthers, K. C., Hovenkamp, E., Niesters, H. G., Osterhaus, A. D., Miedema, F. & van Oers, M. H. (2002c). Absolute level of Epstein-Barr virus DNA in human immunodeficiency virus type 1 infection is not predictive of AIDS-related non-Hodgkin lymphoma. *J Infect Dis* **186**, 405-409.

van Noesel, C. J., Gruters, R. A., Terpstra, F. G., Schellekens, P. T., van Lier, R. A. & Miedema, F. (1990). Functional and phenotypic evidence for a selective loss of memory T cells in asymptomatic human immunodeficiency virus-infected men. *J Clin Invest* **86**, 293-299.

Veazey, R. & Lackner, A. (2003). The mucosal immune system and HIV-1 infection. *AIDS Rev* **5**, 245-252.

Veazey, R. S., DeMaria, M., Chalifoux, L. V., Shvetz, D. E., Pauley, D. R., Knight, H. L., Rosenzweig, M., Johnson, R. P., Desrosiers, R. C. & Lackner, A. A. (1998). Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* **280**, 427-431.

Veazey, R. S., Mansfield, K. G., Tham, I. C., Carville, A. C., Shvetz, D. E., Forand, A. E. & Lackner, A. A. (2000). Dynamics of CCR5 expression by CD4(+) T cells in lymphoid tissues during simian immunodeficiency virus infection. *J Virol* **74**, 11001-11007.

Waldrop, S. L., Pitcher, C. J., Peterson, D. M., Maino, V. C. & Picker, L. J. (1997). Determination of antigen-specific: Memory/effector CD4+ T cell frequencies by flow cytometry - Evidence for a novel, antigen- specific homeostatic mechanism in HIV-associated immunodeficiency. *J Clin Invest* **99**, 1739-1750.

Walker, B. D., Chakrabarti, S., Moss, B., Paradis, T. J., Flynn, T., Durno, A. G., Blumberg, R. S., Kaplan, J. C., Hirsch, M. S. & Schooley, R. T. (1987). HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature* **328**, 345-348.

Walker, C. M., Moody, D. J., Stites, D. P. & Levy, J. A. (1986). CD8+ Lymphocytes Can Control HIV Infection in Vitro by Suppressing Virus Replication. *Science* **234**, 1563-1566.

Wei, X. P., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H., Saag, M. S. & Shaw, G. M. (1995). Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**, 117-122.

Weimer, R., Schweighoffer, T., Schimpf, K. & Opelz, G. (1989). Helper and suppressor T-cell function in HIV-infected hemophilia patients. *Blood* **74**, 298-302.

Weinberg, J. B., Matthews, T. J., Cullen, B. R. & Malim, M. H. (1991). Productive human immunodeficiency virus type 1 (HIV-1) infection of nonproliferating human monocytes. *J Exp Med* **174**, 1477-1482.

Weiss, L., Roux, A., Garcia, S., Demouchy, C., Haeffnercavaillon, N., Kazatchkine, M. D. & Gougeon, M. L. (1998). Persistent expansion, in a human immunodeficiency virus-infected person, of V beta-restricted CD4(+)CD8(+) T lymphocytes that express cytotoxicity-associated molecules and are committed to produce interferon-gamma and tumor necrosis factor-alpha. *J Infect Dis* **178**, 1158-1162.

Whittle, H. C., Dossetor, J., Oduloju, A., Bryceson, A. D. & Greenwood, B. M. (1978). Cell-mediated immunity during natural measles infection. *J Clin Invest* **62**, 678-684.

Wilkie, G. M., Taylor, C., Jones, M. M., Burns, D. M., Turner, M., Kilpatrick, D., Amlot, P. L., Crawford, D. H. & Haque, T. (2004). Establishment and characterization of a bank of cytotoxic T lymphocytes for immunotherapy of epstein-barr virus-associated diseases. *J Immunother* **27**, 309-316.

Wolthers, K. C., Wisman, G. B. A., Otto, S. A., De Roda Husman, A.-M., Schaft, N., de Wolf, F., Goudsmit, J., Coutinho, R. A., van der Zee, A. G. J., Meyaard, L. & Miedema, F. (1996). T cell telomere length in HIV-1 infection: no evidence for increased CD4+ T cell turnover. *Science* **274**, 1543-1547.

Wong, J. K., Hezareh, M., Gunthard, H. F., Havlir, D. V., Ignacio, C. C., Spina, C. A. & Richman, D. D. (1997) . Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science* **278**, 1291-1295.

Wyatt, R. & Sodroski, J. (1998). The HIV-1 envelope glycoproteins: Fusogens, antigens, and immunogens. *Science* **280**, 1884-1888.

Xu, X. N., Screaton, G. R., Gotch, F. M., Dong, T., Tan, R. S., Almond, N., Walker, B., Stebbings, R., Kent, K., Nagata, S., Stott, J. E. & McMichael, A. J. (1997). Evasion of cytotoxic T lymphocyte (CTL) responses by Nef- dependent induction of Fas ligand (CD95L) expression on simian immunodeficiency virus-infected cells. *J Exp Med* **186**, 7-16.

Yagi, N., Satonaka, K., Horio, M., Shimogaki, H., Tokuda, Y. & Maeda, S. (1996). The role of DNase and EDTA on DNA degradation in formaldehyde fixed tissues. *Biotech Histochem* **71**, 123-129.

Yang, L. P., Riley, J. L., Carroll, R. G., June, C. H., Hoxie, J., Patterson, B. K., Ohshima, Y., Hodes, R. J. & Delespesse, G. (1998). Productive infection of neonatal CD8(+) T lymphocytes by HIV-1. *J Exp Med* **187**, 1139-1144.

Yang, O. O., Kalams, S. A., Rosenzweig, M., Trocha, A., Jones, N., Koziel, M., Walker, B. D. & Johnson, R. P. (1996). Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes. *J Virol* **70**, 5799-5806.

Zack, J. A., Arrigo, S. J., Weitsman, S. R., Go, A. S., Haislip, A. & Chen, I. S. (1990). HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* **61**, 213-222.

Zerhouni, B., Nelson, J. A. & Saha, K. (2004). Isolation of CD4-independent primary human immunodeficiency virus type 1 isolates that are syncytium inducing and acutely cytopathic for CD8+ lymphocytes. *J Virol* **78**, 1243-1255.

Zhang, D., Shankar, P., Xu, Z., Harnisch, B., Chen, G., Lange, C., Lee, S. J., Valdez, H., Lederman, M. M. & Lieberman, J. (2003). Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic. *Blood* **101**, 226-235.

Zhang, J., Gupta, A., Dave, R., Yimen, M., Zerhouni, B. & Saha, K. (2001). Isolation of primary HIV-1 that target CD8+ T Lymphocytes using CD8 as a receptor. *Nat Med* **7**, 65-72.

Zhang, Z. Q., Notermans, D. W., Sedgewick, G., Cavert, W., Wietgrefe, S., Zupancic, M., Gebhard, K., Henry, K., Boies, L., Chen, Z. M., Jenkins, M., Mills, R., Mcdade, H., Goodwin, C., Schuwirth, C. M., Danner, S. A. & Haase, A. T. (1998). Kinetics of CD4+ T cell repopulation of lymphoid tissues after treatment of HIV-1 infection. *Proc Natl Acad Sci USA* **95**, 1154-1159.

Zloza, A., Sullivan, Y. B., Connick, E., Landay, A. L. & Al Harthi, L. (2003). CD8+ T cells that express CD4 on their surface (CD4dimCD8bright T cells) recognize an antigen-specific target, are detected in vivo, and can be productively infected by T-tropic HIV. *Blood* **102**, 2156-2164.

Zuckermann, F. A. & Husmann, R. J. (1996). Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. *Immunology* **87**, 500-512.

Appendices

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High Levels of Human Immunodeficiency Virus Infection of CD8 Lymphocytes Expressing CD4 In Vivo

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Human immunodeficiency virus (HIV)-infected CD8 lymphocytes have been reported in vivo, but the mechanism of infection remains unclear. Experiments using the thy/hu mouse model support export of intrathymically infected CD8 precursors, while recent in vitro data suggest that mature CD8 lymphocytes upregulate CD4 upon activation (generating a $CD8^{bright}$ $CD4^{dim}$ phenotype) and are susceptible to HIV infection. To determine whether these mechanisms operate in vivo and to assess their relative importance in the generation of circulating HIV-infected CD8 lymphocytes, we quantified HIV long terminal repeat (LTR) DNA in $CD8^+$ $CD4^-$ and $CD8^{bright}$ $CD4^{dim}$ lymphocytes isolated from HIV-infected individuals by fluorescence-activated cell sorting. HIV infection of CD8 lymphocytes was demonstrated in 17 of 19 subjects, with a significant inverse relationship between level of infection and CD4 lymphocyte count ($R = -0.73$; $P < 0.001$). The level of HIV infection of $CD8^{bright}$ $CD4^{dim}$ lymphocytes was significantly higher (median, 1,730 HIV LTR copies/10^6 cells; $n = 9$) than that of $CD8^+$ $CD4^-$ lymphocytes (undetectable in seven of nine individuals; $P < 0.01$) and approached that of CD4 lymphocytes from the same individuals (median, 3,660 HIV LTR copies/10^6 cells). $CD8^{bright}$ $CD4^{dim}$ lymphocytes represented 0.8 to 3.3% of total CD8 lymphocytes and were most prevalent in the memory subset. Thus, HIV-infected CD8 lymphocytes commonly circulate in HIV-infected individuals and are generated through infection of activated CD8 lymphocytes rather than through export of intrathymically infected precursors. The high level of infection of $CD8^{bright}$ $CD4^{dim}$ lymphocytes could have a direct role in the decline in CD8 lymphocyte function that accompanies HIV disease progression.

Although the major cellular target of human immunodeficiency virus type 1 (HIV-1) is the CD4 lymphocyte, there is increasing in vitro (8, 17, 33, 35, 36) and in vivo (3, 8, 14, 20, 21, 29, 30) evidence that CD8 lymphocytes are also susceptible to HIV-1 infection. It is well established that in chronic HIV infection, deterioration in CD8 lymphocyte function accompanies progression to symptomatic disease (12, 19, 24). While this is generally ascribed to lack of CD4 lymphocyte help or to soluble viral factors, it is also possible that direct infection of CD8 lymphocytes by HIV may contribute to their functional decline. In addition to the potential immunosuppressive effect, infection of CD8 lymphocytes generates a reservoir of circulating HIV-infected cells, with implications for virus dissemination and antiviral escape (25).

Various mechanisms for HIV entry into CD8 lymphocytes have been proposed, including cell-to-cell transfer (5), selection of CD8-tropic HIV variants (34, 35), or entry through a conventional CD4-dependent pathway. Two opportunities for CD4-dependent HIV entry into CD8 lymphocytes have been identified. The first occurs during intrathymic CD8 lymphocyte development and the second upon activation of the mature CD8 lymphocyte. Intrathymic CD8 lymphocyte precursors express CD4 and are susceptible to HIV infection in vitro (7). In the thy/hu mouse model, maturation and export of HIV-infected lymphocyte precursors have been shown to generate circulating HIV-infected naïve lymphocytes (4). Such cells could survive in a resting state for years, acting as a reservoir of provirus unaffected by antiretroviral agents.

A number of investigators have shown that activation of mature CD8 lymphocytes in vitro leads to CD4 expression on the cell surface (8, 17, 31), and this mechanism is thought to generate the circulating $CD8^{bright}$ $CD4^{dim}$ lymphocytes observed in vivo (14). HIV infection of in vitro-activated CD8 lymphocytes has been demonstrated (8, 17, 35, 36). Infection of CD8 lymphocytes by this mechanism would target infection to cells responding to antigens, and thus, infection of a relatively small number of cells could have a disproportionately high impact on immune function.

To determine the relative importance of these different mechanisms of infection in the generation of circulating HIV-infected CD8 lymphocytes in vivo, we used PCR to quantify HIV long terminal repeat (LTR) DNA in CD8 lymphocyte subsets sorted on the basis of differentiation phenotype or CD4 expression. This method detects HIV provirus and HIV preintegration complexes, with each HIV DNA genome containing 2 LTR copies. It provides a measure of the level of HIV infection within a given cell population. Differentiation phenotype was defined in terms of CD45RA and CD27 expression, allowing cells to be divided into antigen-naïve, memory, and effector subsets. Intrathymic infection would be expected to generate HIV-infected naïve CD8 lymphocytes with no pref-

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TABLE 1. Clinical profiles of HIV-infected subjects[a]

Study subject[b]	CD4 count/μl	Plasma viral load/ml	Combination antiretroviral therapy[c]
1	2	75,000	No
2	17	750,000	Yes
3	39	309	Yes
4	51	23,100	No
5	66	75,000	Yes
6	117	75,000	Yes
7	123	330,000	Yes
8	296	470	Yes
9	328	<50	Yes
10	334	<50	No
11	339	14,900	Yes
12	364	304	No
13	385	153	Yes
14	393	403	Yes
15	396	862	No
16	509	<50	Yes
17	531	<50	Yes
18	647	13,400	No
19A	824	1,900	Yes
19B	887	<50	Yes

[a] Ranked by CD4 count (from low to high).
[b] Identification numbers of long-term nonprogressors are italicized. Blood samples were drawn from subject 19 on two occasions.
[c] Yes, subject was prescribed at least three antiretroviral agents at the time when the sample was drawn.

erence for CD8 lymphocytes expressing CD4. In contrast, infection during activation would be expected to generate HIV-infected, antigen-experienced CD8 lymphocytes, with higher levels of infection in CD8 lymphocytes expressing CD4.

We also used four-color flow cytometry to investigate the differentiation phenotype of CD4-expressing CD8 lymphocytes isolated from HIV-infected subjects. CD8 lymphocytes induced to express CD4 in vitro have been shown to have cytokine expression profiles in keeping with a significant role in immune function (18, 36), but little is known about the relationship between CD4 expression and progression from naïve to memory or effector status in vivo.

MATERIALS AND METHODS

Subjects. Twenty 30- to 50-ml blood samples were drawn from 19 HIV-infected individuals attending health care services in Scotland (Table 1). Because each sample contained insufficient CD8 lymphocytes to assess HIV infection of all the cell subsets of interest, the samples were divided into two groups. CD8 lymphocytes in the first group were sorted on the basis of CD4 expression (n = 9), and those in the second were sorted on the basis of differentiation phenotype (n = 11). Subjects were selected in such a way as to ensure a range of disease stages, from asymptomatic to advanced AIDS, in each group. Three individuals were long-term nonprogressors (defined as persons who had maintained a CD4 count above 300 without antiretroviral therapy despite more than 10 years of infection), and 13 were receiving combination antiretroviral therapy at the time of sampling. The mean age of the subjects was 39 (range, 29 to 54).

Isolation of lymphocyte populations. Each blood sample was taken into tubes containing EDTA. Peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation over Histopaque (Sigma Diagnostics, St. Louis, Mo.) and washed twice in phosphate-buffered saline (Invitrogen, Paisley, United Kingdom), and lymphocyte subsets were isolated. In order to achieve very high cell population purity, the populations of interest were first enriched by using immunomagnetic technology and then isolated by fluorescence-activated cell sorting (FACS). This two-stage process was performed for all subjects except subject 10, for whom cell sorting was performed directly on PBMCs.

Where CD8 lymphocytes were to be separated by differentiation phenotype, cell populations were enriched by the following steps: (i) γδ T lymphocytes were removed by negative selection using a magnetic cell sorting (MACS) γδ T-lymphocyte isolation kit (Miltenyi Biotec Ltd. [UK], Bisley, United Kingdom); (ii) from the γδ T-lymphocyte-negative population, CD8 lymphocytes were enriched by using MACS CD8 microbeads (Miltenyi Biotec); (iii) from the CD8-negative population, CD4 lymphocytes were enriched by using MACS CD4 microbeads (Miltenyi Biotec). Where CD8 lymphocytes were to be separated into populations on the basis of CD4 expression, steps ii and iii of this protocol were used.

Enriched populations were incubated for 30 min at 4°C with appropriate fluorescent dye-conjugated monoclonal antibodies (Table 2). Cells were then fixed in 0.75% paraformaldehyde (Sigma Diagnostics) at 4°C for 1 h, after which they were resuspended in phosphate-buffered saline and stored for a maximum of 4 days prior to flow sorting. High-purity CD4 lymphocytes and CD8 lymphocyte subpopulations were then isolated by using a FACSVantage (Becton Dickinson, Crawley, United Kingdom) flow sorter (Fig. 1). To ensure that the sorted CD8 lymphocytes were not contaminated by minor populations of other cell types expressing CD8 (such as CD4 lymphocytes and NK cells), the anti CD8 monoclonal antibody used was directed against the β chain of the CD8 molecule, which has been shown to define true CD8 lymphocytes (13). In sorting of the $CD8^{bright}$ $CD4^{dim}$ population, a tight lymphocyte gate and a low flow rate (approximately 2,000 events/s) were used in order to avoid cell aggregates and to minimize coincidence error.

Nucleic acid extraction and HIV DNA quantification. DNA was isolated from the cell subsets by phenol-chloroform extraction and ethanol precipitation, and the DNA concentration was measured by using a GeneQuant II spectrophotometer (Amersham Biosciences, Cambridge, United Kingdom). Real-time PCR was used to quantify HIV LTR DNA copies per microgram of DNA, and the copy number was then confirmed at limiting dilution. The real-time PCR was performed using a nested approach with a conventional primary reaction and a real-time secondary reaction. The primary reaction used primers GRAACC CACTGCTTAASSCTCAA (sense) and AAGCCGAGYCCTGCGTCGAGAG (antisense) (5′ base positions 506 and 686 of the HXB2 genome) with an annealing temperature of 55°C and ran for 18 cycles. The secondary reaction used primers CTCAATAAAGCTTGCCTTGAG (sense) and TGTTCGGGCGCCA CTGCTAGAGA (antisense) (5′ base positions 524 and 626 of the HXB2 ge-

TABLE 2. Monoclonal antibodies used to stain cell populations for FACS and flow cytometry

Cell population stained	Fluorescent dye-conjugated mouse anti-human monoclonal antibody[a]	Clone	Company
CD8 lymphocytes to be separated by differentiation phenotype and PBMCs for assessment of differentiation phenotype of CD8 lymphocytes expressing CD4	CD27 FITC	M-T271	BD Biosciences
	CD8β chain PE	2ST8.5H7	Coulter Immunotech
	CD45RA Cy-chrome	HI100	Pharmingen
	CD4-APC	RPA-T4	BD Biosciences
CD8 lymphocytes to be separated by CD4 expression	CD8β chain PE	2ST8.5H7	Coulter Immunotech
	CD4 Cy-chrome	RPA-T4	BD Biosciences
CD4 lymphocytes	CD3 PE	UCHT1	BD Biosciences
	CD4 Cy-chrome	RPA-T4	BD Biosciences

[a] FITC, fluorescein isothiocyanate isomer 1; PE, phycoerythrin; APC, allophycocyanin.

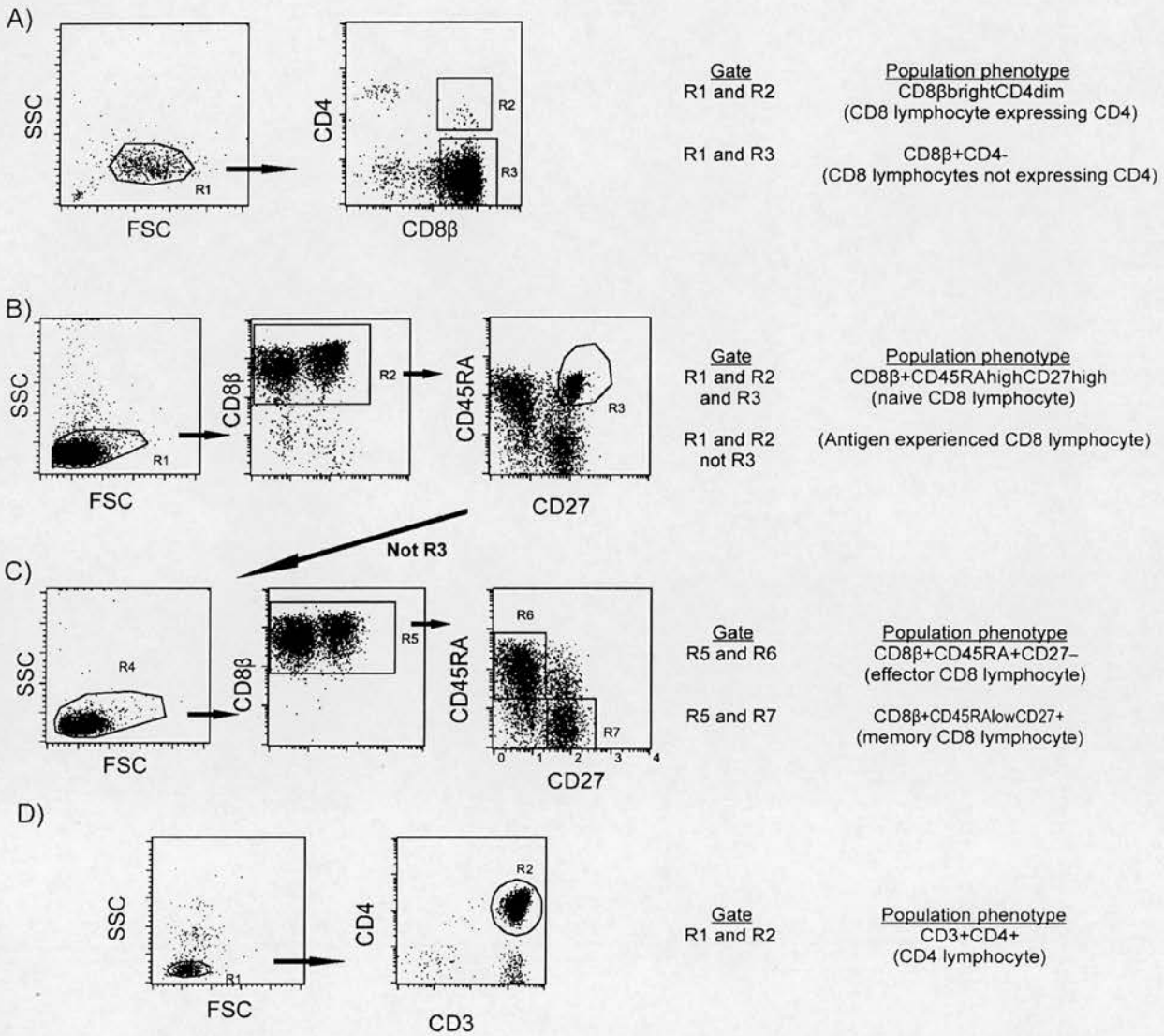

FIG. 1. FACS of CD4 lymphocytes and CD8 lymphocyte subsets. (A) CD8 lymphocytes sorted on the basis of CD4 expression; (B and C) CD8 lymphocytes sorted on the basis of differentiation phenotype; (D) CD4 lymphocytes. Sorting on the basis of differentiation phenotype was performed in two stages: first, enriched CD8 lymphocytes were sorted into antigen-naïve and antigen experienced subsets (B); then a portion of the antigen-experienced subset was retained for HIV DNA analysis, and the remainder was further sorted into memory and effector subtypes (C). FSC, forward scatter; SSC, side scatter.

nome) (obtained from Oswell, Southampton, United Kingdom) and hybridization probes LCRed705-ACTCTGGTARCTAGAGATCCCTCAGA-phosphate and AAGTAGTGTGTGCCCGTCTGTTGT-fluorescein (Tib Molbiol, Berlin, Germany) and was performed on a Light Cycler (Roche, Mannheim, Germany) annealing at 55°C, with external standards. This method was sensitive to a single copy, as assessed against National Institute for Biological Standards and Control (NIBSC) HIV-1 DNA standards (provided by the European Union Programme EVA/MRC Centralised Facility for AIDS Reagents, NIBSC, Potters Bar, Hertfordshire, United Kingdom [grants QLK2-CT-1999-00609 and GP828102]; donated by J. Bootman), and was validated against serial dilutions of cloned full-length HIV-1.

Limiting-dilution PCR was then performed to confirm the estimated LTR copy number by using published Pan-LTR primers and thermocycling conditions (14). Ten replicate reactions were performed at the DNA concentration expected to contain 0.5 copy per reaction. Further dilutions were performed if necessary to produce replicates containing both positive and negative results. HIV DNA load and standard error were then estimated by using the QUALITY program (27) and were expressed as LTR DNA copies per 10^6 cells, assuming 6.6 μg of DNA per 10^6 cells. For samples where only a single positive replicate was generated due to limited availability of viral DNA, no standard error is provided. The correlation between the original real-time PCR estimate and the final result provided by limiting dilution was 0.79 ($P < 0.001$) (Spearman's rank correlation). This DNA extraction and LTR quantification protocol was found to have high reproducibility when tested on five replicate samples of paraformaldehyde-fixed PBMCs from an HIV-infected subject (mean, 60 LTR DNA copies/10^6 cells; range, 26 to 100).

Purity of cell subsets and calculation of attributable HIV DNA loads. The level of CD4 lymphocyte contamination of CD8 lymphocyte subsets (defined as the percentage of cells that were $CD4^+$ $CD8\beta^-$) was assessed by flow cytometry and was found to be low (mean, 0.05% [Tables 3 and 4]). For subsets with adequate cell numbers (naïve and experienced CD8 lymphocytes and $CD8\beta^+$ $CD4^-$ lymphocytes), at least 1,000 (mean, 4,880) ungated events were assessed. Within subjects, the purity of memory and effector populations was taken to be equivalent to that of antigen-experienced populations. For populations of rare cells

TABLE 3. Levels of CD4 lymphocyte contamination of $CD8\beta^+$ $CD4^-$ and $CD8\beta^{bright}$ $CD4^{dim}$ lymphocytes

Study subject	% CD4 lymphocyte contamination[a] of:	
	$CD8\beta^+$ $CD4^-$ lymphocytes	$CD8\beta^{bright}$ $CD4^{dim}$ lymphocytes
1	$<5 \times 10^{-3}$	NA
5	$<9 \times 10^{-4}$	NA
6	0.1	0.56
7	0.01	NA
11	0.17	0.20
13	0.01	NA
14	0.13	2.44
15	0.3	NA
19B	<0.11	<0.74
Median	0.1	0.65

[a] NA, not available.

($CD8^{bright}$ $CD4^{dim}$), sufficient cells were available to assess purity in four subjects. At least 500 (mean, 860) events were assessed, and due to the high ratio of background to cellular events, events falling outside the lymphocyte gate (defined on light scatter properties) were excluded. Where purity was not measured directly, the purity of $CD8^{bright}$ $CD4^{dim}$ lymphocytes was taken to be equivalent to that of $CD8\beta^+$ $CD4^-$ lymphocytes. Where no purity data were available (subjects 9 and 17), a level of 0.2%, which represents the 75th percentile of the available CD4 contamination data, was used. Microscopic examination of sorted $CD8^{bright}$ $CD4^{dim}$ lymphocytes from one subject confirmed that they represented a single cell suspension free from aggregates.

For each CD8 lymphocyte subset, the HIV DNA load attributable to CD4 lymphocyte contamination was calculated from the cell population purity data and the CD4 lymphocyte HIV DNA load. This figure was then subtracted from the observed HIV DNA load in the CD8 lymphocyte subset to give the HIV DNA load attributable to CD8 lymphocytes. Where CD8 lymphocytes were found to be infected, the HIV DNA load observed in the CD8 lymphocyte subset was >5 times that attributable to CD4 contamination in all but three cases. All HIV DNA loads given in Results for CD8 lymphocyte subsets are attributable to the CD8 lymphocytes.

Distribution of CD8 lymphocytes expressing CD4. The differentiation phenotype, in terms of CD45RA and CD27 expression (Fig. 1), of CD8 lymphocytes expressing CD4 was assessed for 13 subjects by using a FACScalibur flow cytometer (Becton Dickinson). For seven subjects this assessment was performed on sorted naïve and experienced CD8 lymphocyte populations, while for the remainder appropriately stained PBMCs were used (Table 2). Populations were gated according to the differentiation phenotypes given (Fig. 1), and the percentage of CD8 lymphocytes expressing CD4 in each population was determined. A tight lymphocyte gate was used to exclude cell aggregates.

RESULTS

Extent of HIV infection of CD8 lymphocytes. To assess the level of HIV infection of CD8 lymphocytes in subjects with a range of disease stages, HIV LTR copies were quantified in CD8 lymphocytes isolated from HIV-infected subjects. HIV infection of CD8 lymphocytes was demonstrated in 17 out of 19 subjects. The level of infection was low (median, 16 HIV LTR DNA copies per 10^6 CD8 lymphocytes; range, undetectable to 436 [Fig. 2A]), significantly lower than that of CD4 lymphocytes from the same subjects (median, 3,660 LTR DNA copies/10^6 cells; range, 16 to 25,512 [$P < 0.001$ by the Wilcoxon signed rank test] [Fig. 2A]). The possibility that the viral DNA found within the CD8 lymphocyte subsets represented contamination from CD4 lymphocytes was excluded by determining the purity of the CD8 lymphocyte populations and calculating attributable HIV DNA loads (see Materials and Methods). No significant correlation was found between HIV DNA loads in CD4 and CD8 lymphocytes (Spearman's correlation coefficient, 0.3; $P > 0.1$).

There was an inverse correlation between CD4 lymphocyte counts and CD8 lymphocyte HIV DNA loads (Spearman's correlation coefficient [R], −0.73; $P < 0.001$ [Fig. 3A]), indicating a progressive increase in the prevalence of infected CD8 lymphocytes with advancing disease. In individuals with advanced disease, the higher prevalence of infected CD8 lymphocytes, together with dwindling CD4 lymphocyte numbers, increased the contribution of CD8 lymphocytes to the total circulating HIV DNA load. Thus, for the individuals with the lowest CD4 lymphocyte counts, CD8 lymphocytes contributed more than 25% of the total HIV DNA load in circulating lymphocytes (Fig. 3B). In contrast, the CD8 lymphocytes of long-term nonprogressors appear relatively resistant to infection, with virtually no LTR copies detected.

Phenotype of HIV-infected CD8 lymphocytes. In order to determine the relative contributions of intrathymic infection versus infection of activated cells in the generation of circulating HIV-infected CD8 lymphocytes, HIV LTR DNA was quantified in subsets of CD8 lymphocytes. For the first nine subjects, CD8 lymphocyte subsets were divided on the basis of CD4 expression (Fig. 2B). CD8 lymphocytes expressing CD4 ($CD8^{bright}$ $CD4^{dim}$) were found to be infected in seven out of nine subjects (78%) with relatively high levels of infection (median, 1,730 LTR DNA copies/10^6 cells; range, undetectable to 7,902), approaching those in the CD4 lymphocytes of the same nine subjects (Fig. 4A). In contrast, only two of nine subjects (22%) showed infection of $CD8\beta^+$ $CD4^-$ lymphocytes, and this was at low levels (10 and 4 LTR DNA copies/10^6 cells). The increase in HIV DNA load in $CD8^{bright}$ $CD4^{dim}$ lymphocytes compared to that in $CD8\beta^+$ $CD4^-$ lymphocytes was significant ($P < 0.01$ where undetectable values were assigned a value half the lower limit of detection, and $P < 0.05$ where undetectable values were assigned a value of zero, by the Wilcoxon signed rank test).

For the remaining 11 subjects, levels of HIV infection were compared in CD8 lymphocyte subsets divided on the basis of differentiation phenotype (Fig. 2C and 4B). Infection levels

TABLE 4. Levels of CD4 lymphocyte contamination of naïve and antigen-experienced CD8 lymphocytes

Study subject	% CD4 lymphocyte contamination[a] of:	
	Naïve CD8 lymphocytes	Antigen-experienced CD8 lymphocytes
2	0.2	<0.01
3	<0.03	NA
4	<0.03	0.1
8	<0.03	0.03
9	NA	NA
10	0.41[b]	NA
12	<0.05	<0.05
16	0.04	0.03
17	NA	NA
18	0.1	0.79
19A	0.02	0.04
Median	0.04	0.03

[a] NA, not available.
[b] PBMCs from subject 10 were not enriched prior to FACS.

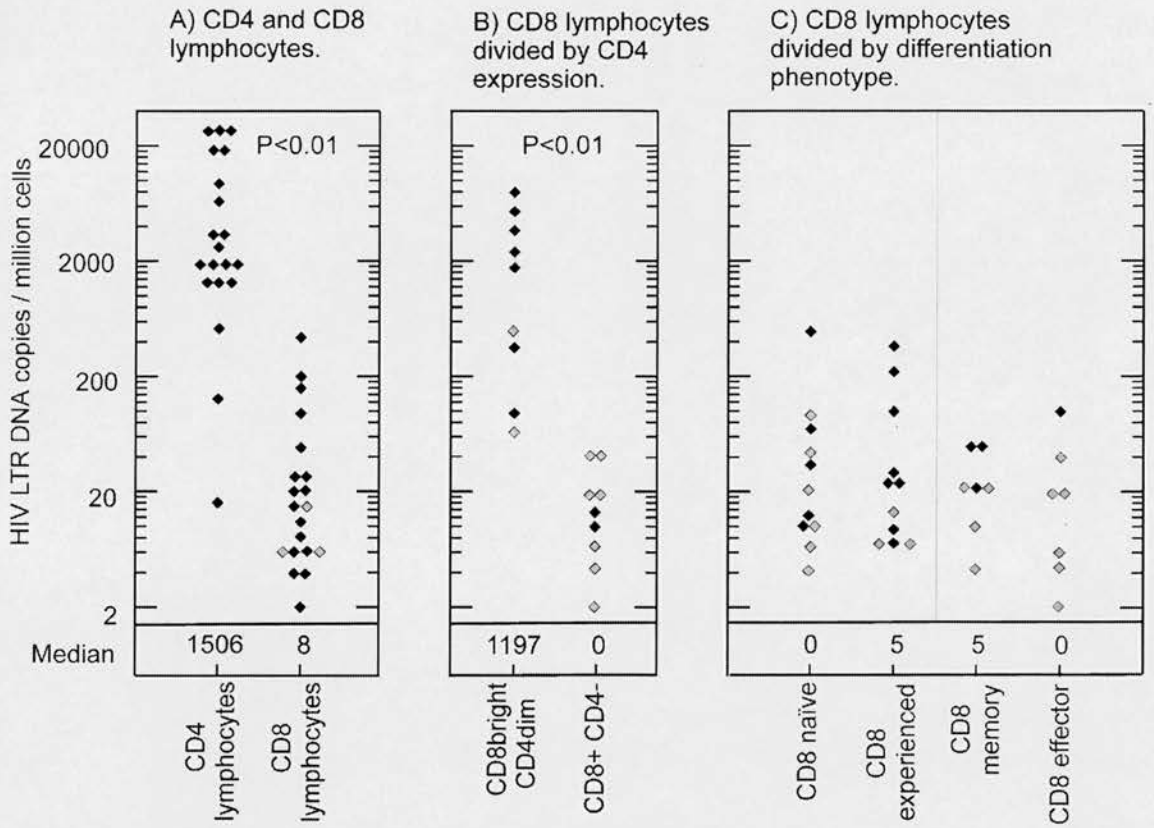

FIG. 2. Levels of HIV infection in CD4 lymphocytes and CD8 lymphocyte subsets. (A) CD4 and CD8 lymphocytes; (B) $CD8\beta^{bright}$ $CD4^{dim}$ and $CD8\beta^{+}$ $CD4^{-}$ lymphocytes; (C) CD8 lymphocytes divided by differentiation phenotype. Solid diamonds, attributable HIV DNA load. Shaded diamonds, samples where no virus was detected; the value given is half the lower limit of detection, which varies with the number of cells available for analysis. *P* values shown were calculated using the Wilcoxon signed rank test.

were generally quite low (range, <3 to 274 LTR DNA copies/10^6 cells), and although HIV DNA was more frequently detected in antigen-experienced than in antigen-naïve populations, there was no significant difference in HIV DNA loads ($P = 0.7$ by the Wilcoxon signed rank test). Further subdivision of the antigen-experienced cells into memory and effector subsets was performed for four of the subjects with detectable HIV DNA. Of these, three demonstrated infection in the memory subset and one demonstrated infection in the effector subset (Fig. 2C), again with no significant difference in viral DNA loads.

Prevalence and differentiation status of CD8 lymphocytes expressing CD4. In order to investigate the relationship between CD8 lymphocyte differentiation and CD4 expression in vivo, we used four-color flow cytometry to assess CD4, CD8β, CD45RA, and CD27 expression in 13 HIV-infected subjects. Distinct $CD8^{bright}$ $CD4^{dim}$ populations were detected in all study subjects, and the density of CD4 expression on $CD8^{bright}$ $CD4^{dim}$ lymphocytes was approximately half (mean, 0.4; range, 0.3 to 0.6) that on true CD4 lymphocytes. The proportion of CD8 lymphocytes found to express CD4 was between 0.8 and 3.3% and was not found to correlate with CD4 or CD8 lymphocyte counts.

In keeping with the hypothesis of CD4 expression following activation, the vast majority of $CD8^{bright}$ $CD4^{dim}$ lymphocytes had an antigen-experienced phenotype (mean, 92%; range, 47 to 99%). Interestingly, most of the antigen-experienced $CD8^{bright}$ $CD4^{dim}$ lymphocytes displayed a memory (mean, 75%; range, 47 to 96%) as opposed to an effector (mean, 12%; range, 0 to 31%) phenotype. This preponderance of antigen-experienced and memory phenotypes is not simply a reflection of the distribution of phenotypes in the total CD8 lymphocyte population, because the proportion of CD8 lymphocytes expressing CD4 was significantly greater in the antigen-experienced population than in the antigen-naïve population ($P < 0.05$ by paired *t* test) and significantly greater in the memory subset than in the effector subset ($P < 0.01$ by paired *t* test [Fig. 5]).

The differentiation phenotype of $CD8^{bright}$ $CD4^{dim}$ lymphocytes was also assessed in three healthy volunteers. All three demonstrated a differentiation pattern similar to that observed in the majority of HIV-infected subjects, with the greatest proportion of CD8 lymphocytes expressing CD4 observed in the memory subset (range, 3.8 to 6.3%) and markedly less found in the effector (range, 1.0 to 2.0%) and naïve (range, 1.1 to 2.8%) subsets.

DISCUSSION

This study confirms that CD8 lymphocytes are infected with HIV in vivo and demonstrates that CD8 lymphocytes expressing CD4 have a high frequency of infection. It is the first study

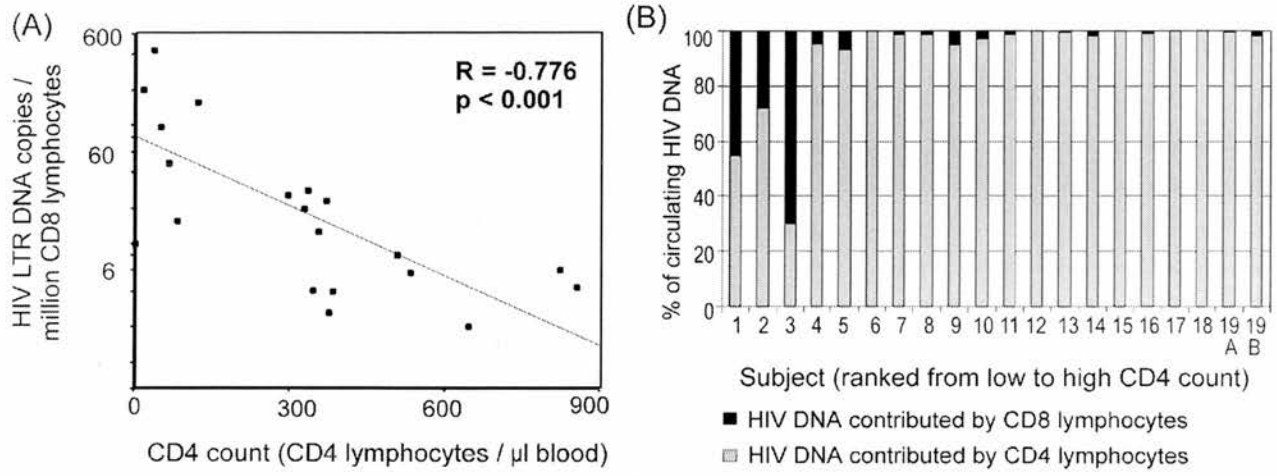

FIG. 3. Increased HIV infection of CD8 lymphocytes with disease progression. (A) Correlation between CD8 lymphocyte HIV DNA load and CD4 count (Spearman's rank correlation coefficient [*R*]). Cases with undetectable proviral loads were given a value of half the lower limit of detection. (B) Relative contributions of CD4 and CD8 lymphocytes to overall circulating HIV DNA loads were calculated from CD4 and CD8 lymphocyte HIV DNA loads and from CD4 and CD8 lymphocyte counts. Undetectable viral loads were given a value of zero.

to correct the level of infection of CD8 lymphocytes for directly measured CD4 lymphocyte contamination and therefore represents a methodological advance over previous studies (14, 21). The high infection levels found in $CD8^{bright}$ $CD4^{dim}$ lymphocytes support the hypothesis that infection of this subset could have a direct role in the AIDS-related decline in CD8 lymphocyte function.

Route of CD8 lymphocyte infection. CD4-dependent HIV infection of CD8 lymphocytes has been demonstrated during intrathymic development (4, 7) and following activation of mature lymphocytes (8, 14, 17, 35). Given that CD8 lymphocyte precursors would be expected to lose CD4 before leaving the thymus, our observation of very low or undetectable viral DNA loads in $CD8\beta^+$ $CD4^-$ lymphocytes implies that this

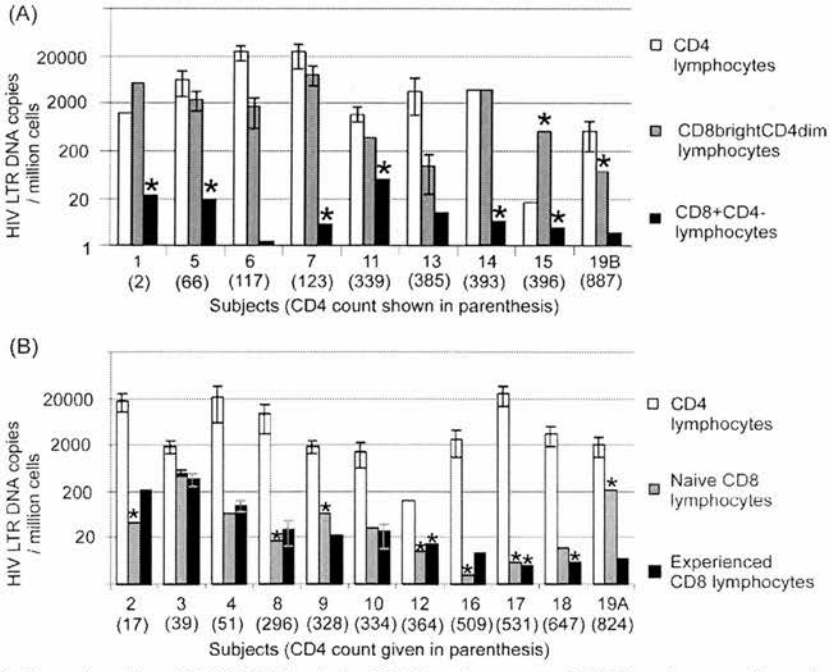

FIG. 4. HIV DNA loads for each subject. HIV DNA loads for CD4 lymphocytes and CD8 lymphocyte subsets for each subject (ordered by ascending CD4 lymphocyte count) are given. (A) CD8 lymphocytes were divided on the basis of CD4 expression; (B) CD8 lymphocytes were divided by differentiation phenotype. Error bars indicate standard errors (see Materials and Methods); they are absent from samples where insufficient cells were available. Stars indicate samples where no virus was detected; the value given is half the lower limit of detection.

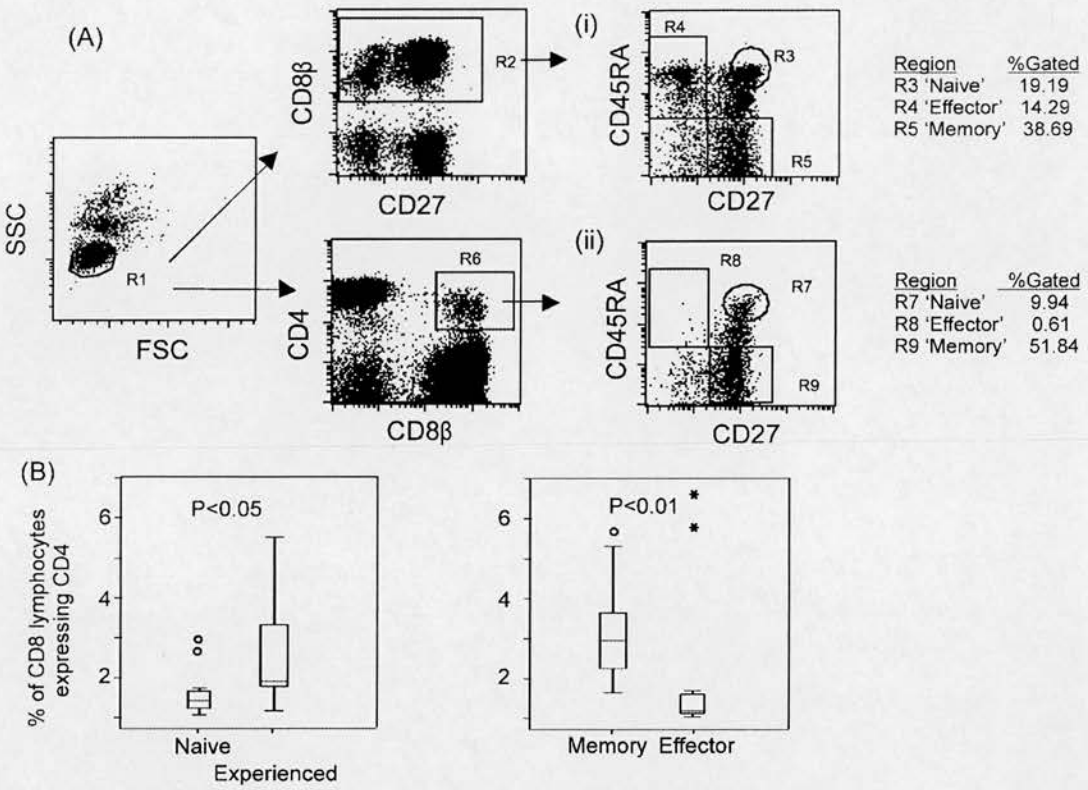

FIG. 5. Phenotype of CD8β^{bright} CD4^{dim} lymphocytes. (A) The differentiation phenotype of CD8β^{bright} CD4^{dim} lymphocytes was assessed in terms of CD45RA and CD27 expression. Dot plots from PBMCs of a representative subject (subject 10) are shown comparing the distribution of all CD8 lymphocytes (i) against that of CD8β^{bright} CD4^{dim} lymphocytes (ii). The lack of expression of CD4 on the CD45RA$^+$ CD27$^-$ (effector) subset is clearly seen. (B) Percentages of CD8 lymphocytes found to express CD4 in antigen-naïve, antigen-experienced, memory, and effector subsets are shown for 13 subjects. Circles, outliers; stars, extreme outliers. *P* values were calculated by using the paired *t* test.

route is contributing little to the circulating HIV-infected CD8 lymphocytes. In contrast, the higher viral DNA levels observed in the CD8^{bright} CD4^{dim} population suggests frequent infection of this subset of mature CD8 lymphocytes. This distribution of HIV DNA is in keeping with previous results from our group in which CD8$^+$ CD4$^+$ cells isolated by immunomagnetic methods were found to contribute a higher proportion to the overall proviral load than their CD4-negative counterparts (14). Similarly, in a recent paper, Brenchley et al. observed that there are 5- to 100-fold more HIV *gag* DNA copies in CD8$^+$ CD4^{dull} lymphocytes than in CD8 lymphocytes lacking CD4 expression (3).

The distribution of HIV DNA between CD8 lymphocytes at sequential stages of differentiation can also provide information regarding the likely route of infection. The use of phenotypic markers to define differentiation stages of CD8 lymphocytes has been a topic of much debate (11). CD27 and CD45RA have been demonstrated to define populations with distinct cytokine profiles, and a differentiation pathway from CD27$^+$ CD45RA$^+$ (antigen naïve) to CD27$^+$ CD45RA$^-$ (memory) and then to CD27$^-$ CD45RA$^+$ (effector) has been proposed (9, 10, 15). Further experiments indicated that >90% of CD27$^+$ CD45RA$^+$ CD8 lymphocytes were "true naïve" as defined by a panel of three further phenotypic markers (6), though early antigen-experienced cells may retain the CD27^{high} CD45RA$^+$ (naïve) phenotype (1). The relationship between differentiation markers and memory versus effector function remains unclear (1), and we therefore recognize that while we retain the labels "memory" and "effector" for the CD27$^+$ CD45RA$^-$ and CD27$^-$ CD45RA$^+$ subsets, respectively, a degree of functional overlap is likely.

Given the observed distribution of HIV DNA in CD8^{bright} CD4^{dim} versus CD8β$^+$ CD4$^-$ lymphocytes, and the evidence that CD4 upregulation on CD8 lymphocytes follows antigen recognition (8, 17, 31), one would expect HIV DNA levels to be relatively high in antigen-experienced CD8 lymphocytes but very low in the antigen-naïve subset. Interestingly, although HIV DNA was more frequently detected in the antigen-experienced populations, it was also demonstrated in the naïve subset for 4 out of 11 subjects. These HIV-infected naïve CD8 lymphocytes are likely to be cells early in the activation process that have upregulated CD4 but have not yet lost their naïve markers. This interpretation is supported by our finding of CD4 expression on lymphocytes with the CD45RA$^+$ CD27^{high} (naïve) phenotype (Fig. 5) and suggests that, at least in some subjects, HIV infection of CD8 lymphocytes is occurring very early in the activation process. Recently published data showed negligible HIV DNA levels in naïve CD8 lymphocyte populations from which cells expressing CD4 had been removed (3), supporting our suggestion that CD4-expressing cells are re-

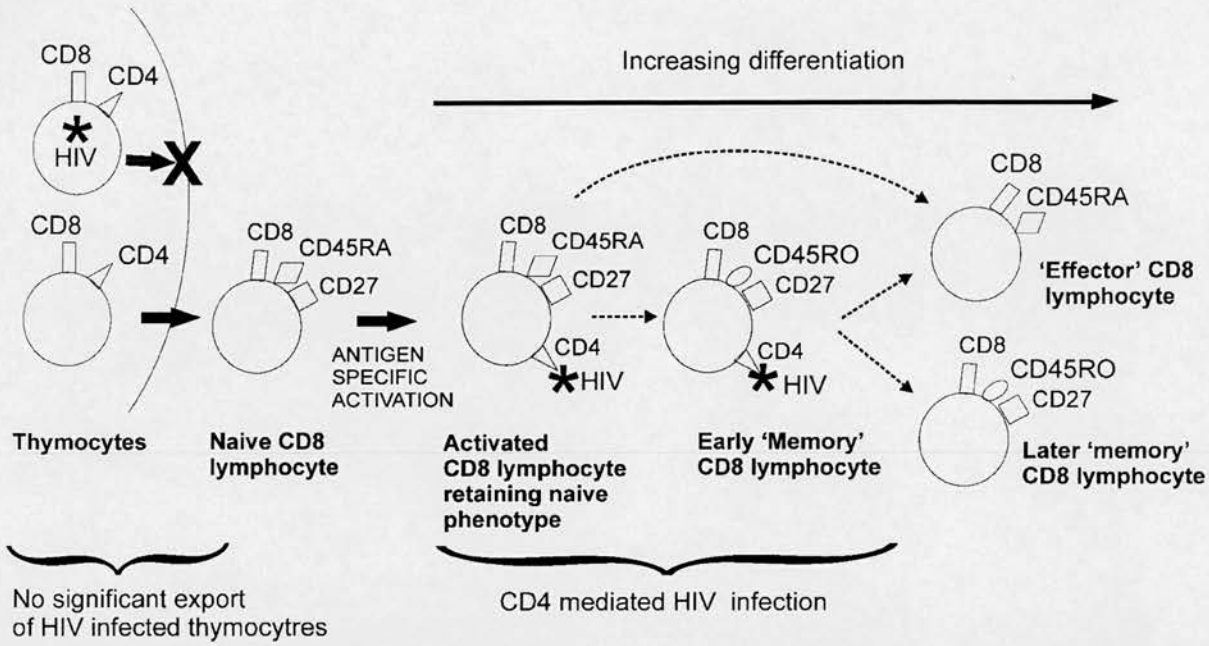

FIG. 6. Proposed infection route of CD8 lymphocytes circulating in vivo. We propose that circulating HIV-infected CD8 lymphocytes are generated through CD4-mediated infection of activated CD8 lymphocytes and that export of HIV-infected CD8 precursors from the thymus occurs rarely if at all. Since only a minority of memory CD8 lymphocytes express CD4, we propose that expression is transient and therefore show a CD4-negative "later " memory CD8 lymphocyte. It is also possible that CD4 is expressed on a stable minority of memory CD8 lymphocytes. Dotted arrows represent possible lineage associations between cell types.

sponsible for the HIV observed in our naïve CD8 lymphocyte populations. Alternatively, the HIV DNA observed in our naïve cell populations may originate in contaminating nonnaïve cells which were excluded in the Brenchley et al. experiment through use of a more stringent definition of naïve cells (3).

The lack of correlation between the level of infection in CD4 and CD8 lymphocytes suggests that, although we propose that HIV enters both cells via the CD4 receptor, different factors influence the frequency of this event in the two cell types. The observation of a clear inverse correlation between CD8 lymphocyte HIV DNA load and CD4 count indicates that disease progression may be one factor that favors CD8 lymphocyte infection. Teasing out the relative importance of the many other host, viral, and therapeutic factors that are likely to influence CD8 lymphocyte infection would be of major value in future studies.

Phenotype of $CD8^{bright}$ $CD4^{dim}$ lymphocytes. Given the finding that $CD8^{bright}$ $CD4^{dim}$ lymphocytes are a major target for HIV infection, a clear understanding of the natural history of these cells in HIV-infected subjects is a priority. In vitro, CD4 is markedly upregulated upon costimulation of naïve CD8 lymphocytes, with CD4 expression accompanying the appearance of activation markers and a change from a naïve to an antigen-experienced phenotype (17, 31). Our observation that the majority of $CD8^{bright}$ $CD4^{dim}$ lymphocytes circulating in vivo have an antigen-experienced phenotype is in keeping with this in vitro finding. CD4 expression was also observed in a small proportion of naïve CD8 lymphocytes, and, as discussed earlier, it is likely that these were recently activated, suggesting that CD4 expression is an early event in the activation process.

Within the antigen-experienced population, CD4 was expressed in a much higher proportion of $CD27^+$ $CD45RA^-$ (memory) than terminally differentiated $CD27^-$ $CD45RA^+$ (effector) CD8 lymphocytes. In the context of the proposed linear pathway of CD8 lymphocyte differentiation from naïve to memory and then to effector function (9, 10), these findings suggest that memory CD8 lymphocytes expressing CD4 either fail to differentiate to effector status or downregulate CD4 expression prior to further differentiation. Failure to differentiate could reflect deletion of CD4-expressing cells through HIV infection; however, the decreased frequency of CD4 expression in effector cells was also observed in three healthy volunteers, suggesting that non-HIV-related mechanisms are involved.

In 2 out of the 13 subjects assessed, an unusually high proportion of effector CD8 lymphocytes was found to express CD4 (indicated as outliers in Fig. 5B). These subjects had the highest overall proportion of $CD8^{bright}$ $CD4^{dim}$ lymphocytes (>4% of all CD8 lymphocytes), and, in contrast to the remaining 11 subjects, the proportion in the effector population was greater than that in the memory population. It is possible that the high levels of $CD8^{bright}$ $CD4^{dim}$ lymphocytes in these subjects reflected acute responses to intercurrent infection (23), and one of the subjects had clinical diagnoses of pneumonia and otitis media at the time of sampling.

Taken together, the HIV DNA load and the CD4 expression data allow us to put forward a model for the route of HIV infection of circulating CD8 lymphocytes (Fig. 6). We propose that export of HIV-infected CD8 lymphocyte precursors from the thymus occurs rarely if at all and that the vast majority of infected CD8 lymphocytes acquire HIV through expression of CD4 during activation. CD4 expression can be induced early in

the activation of naïve cells, and HIV infection can be rapid, in some cases occurring before the loss of naïve markers. The relationship between effector CD8 lymphocytes and both CD4 expression and HIV infection is interesting. Although we isolated effector cells from only four subjects, we have shown that in general, effector cells do not express CD4, and CD4-negative cells are rarely HIV infected. From this we can infer that effector cells are infrequent carriers of HIV. This suggests either that HIV infection of CD8 lymphocytes blocks progression to effector status (through, for example, cell death or interference with differentiation signals) or that CD4 expression occurs only on a subset of activated CD8 lymphocytes which have a different differentiation pathway. The picture is complicated by the two subjects with high levels of CD4 expression on effector lymphocytes and the one subject whose isolated effector CD8 lymphocytes were found to carry HIV DNA. Clearly, the dynamics of CD4 expression and HIV infection of this important subset of CD8 lymphocytes is an area where further study is a priority.

Impact of HIV infection of $CD8^{bright}$ $CD4^{dim}$ lymphocytes. The importance of CD8 lymphocytes in the control of HIV replication and the decline in CD8 lymphocyte function with progression to AIDS have been clearly demonstrated (16, 22, 32). The decline in CD8 lymphocyte function is commonly ascribed to lack of CD4 lymphocyte help and viral escape (reviewed in reference 22), but our group and others (8, 14, 17, 20, 21, 30, 35) have proposed that, in addition to the mechanisms referred to above, infection of CD8 lymphocytes with HIV may directly compromise CD8 lymphocyte function. The present finding of HIV infection in $CD8^{bright}$ $CD4^{dim}$ lymphocytes demonstrates targeted infection of cells responding to antigen. This process could clearly have a significant effect on the immune control of both HIV and opportunistic pathogens and may contribute to the observed correlation between immune activation and poor outcome (2).

Implications for therapeutic advances. As the limitations of current antiviral therapies have become apparent, there has been increasing interest in therapeutic immune activation as a mechanism for achieving improved viral control. Strategies include the use of immune stimulants in conjunction with antiretroviral agents (26) and structured treatment interruptions where antiviral therapy is stopped to allow HIV to replicate and thus stimulate an anti-HIV immune response (28). In the light of our findings, it is clear that such therapies should be developed with caution, with evaluation of their effect on infection of CD8 lymphocytes. Structured treatment interruptions should be viewed as particularly hazardous, because they allow immune activation in the presence of actively replicating HIV.

In conclusion, we have shown that CD8 lymphocytes expressing CD4 contain high levels of HIV DNA in vivo, while CD8 lymphocytes lacking CD4 expression have low or undetectable levels. This finding supports the infection of activated mature CD8 lymphocytes over infection of thymic precursors as the major mechanism responsible for the generation of circulating HIV-infected CD8 lymphocytes. In the future it will be important to investigate factors influencing the expression of CD4 on CD8 lymphocytes and to formally assess the impact of infection of these cells on HIV immunopathogenesis.

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REFERENCES

1. **Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, S. Little, D. V. Havlir, D. D. Richman, N. Gruener, G. Pape, A. Waters, P. Easterbrook, M. Salio, V. Cerundolo, A. J. McMichael, and S. L. Rowland-Jones.** 2002. Memory $CD8^+$ T cells vary in differentiation phenotype in different persistent virus infections. Nat. Med. **8:**379–385.
2. **Appay, V., and S. L. Rowland-Jones.** 2002. Premature ageing of the immune system: the cause of AIDS? Trends Immunol. **23:**580–585.
3. **Brenchley, J. M., B. J. Hill, D. R. Ambrozak, D. A. Price, F. G. Guenaga, J. P. Casazza, J. Kuruppu, J. Yazdani, S. A. Migueles, M. Connors, M. Roederer, D. C. Douek, and R. A. Koup.** 2004. T-cell subsets that harbor human immunodeficiency virus (HIV) in vivo: implications for HIV pathogenesis. J. Virol. **78:**1160–1168.
4. **Brooks, D. G., S. G. Kitchen, C. M. Kitchen, D. D. Scripture-Adams, and J. A. Zack.** 2001. Generation of HIV latency during thymopoiesis. Nat. Med. **7:**459–464.
5. **De Maria, A., S. Colombini, S. Schnittman, and L. Moretta.** 1994. $CD8^+$ cytolytic T lymphocytes become infected in vitro in the process of killing HIV-1-infected target cells. Eur. J. Immunol. **24:**531–536.
6. **De Rosa, S. C., L. A. Herzenberg, L. A. Herzenberg, and M. Roederer.** 2001. Eleven-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. Nat. Med. **7:** 245–248.
7. **De Rossi, A., M. L. Calabro, M. Panozzo, D. Bernardi, B. Caruso, G. Tridente, and L. Chieco-Bianchi.** 1990. In vitro studies of HIV-1 infection in thymic lymphocytes: a putative role of the thymus in AIDS pathogenesis. AIDS Res. Hum. Retrovir. **6:**287–298.
8. **Flamand, L., R. W. Crowley, P. Lusso, S. Colombini-Hatch, D. M. Margolis, and R. C. Gallo.** 1998. Activation of $CD8^+$ T lymphocytes through the T cell receptor turns on CD4 gene expression: implications for HIV pathogenesis. Proc. Natl. Acad. Sci. USA **95:**3111–3116.
9. **Hamann, D., P. Baars, M. H. G. Rep, B. Hooibrink, S. R. Kerkhof-Garde, M. R. Klein, and R. A. W. van Lier.** 1997. Phenotypic and functional separation of memory and effector human $CD8^+$ T cells. J. Exp. Med. **186:** 1407–1418.
10. **Hamann, D., S. Kostense, K. C. Wolthers, S. A. Otto, P. A. Baars, F. Miedema, and R. A. van Lier.** 1999. Evidence that human $CD8^+$ $CD45RA^+$ $CD27^-$ cells are induced by antigen and evolve through extensive rounds of division. Int. Immunol. **11:**1027–1033.
11. **Hamann, D., M. T. Roos, and R. A. van Lier.** 1999. Faces and phases of human CD8 T-cell development. Immunol. Today **20:**177–180.
12. **Hoffenbach, A., P. Langlade-Demoyen, G. Dadaglio, E. Vilmer, F. Michel, C. Mayaud, B. Autran, and F. Plata.** 1989. Unusually high frequencies of HIV-specific cytotoxic T lymphocytes in humans. J. Immunol. **142:**452–462.
13. **Hori, T., X. Paliard, R. de Waal Malefijt, M. Ranes, and H. Spits.** 1991. Comparative analysis of CD8 expressed on mature $CD4^+$ $CD8^+$ T cell clones cultured with IL-4 and that on $CD8^+$ T cell clones: implication for functional significance of CD8β. Int. Immunol. **3:**737–741.
14. **Imlach, S., S. McBreen, T. Shirafuji, C. Leen, J. E. Bell, and P. Simmonds.** 2001. Activated peripheral CD8 lymphocytes express CD4 in vivo and are targets for infection by human immunodeficiency virus type 1. J. Virol. **75:** 11555–11564.
15. **Kern, F., E. Khatamzas, I. Surel, C. Frommel, P. Reinke, S. L. Waldrop, L. J. Picker, and H. D. Volk.** 1999. Distribution of human CMV-specific memory T cells among the CD8pos. subsets defined by CD57, CD27, and CD45 isoforms. Eur. J. Immunol. **29:**2908–2915.
16. **Kersten, M. J., M. R. Klein, A. M. Holwerda, F. Miedema, and M. H. J. van Oers.** 1997. Epstein-Barr virus-specific cytotoxic T cell responses in HIV-1 infection: different kinetics in patients progressing to opportunistic infection or non-Hodgkin's lymphoma. J. Clin. Investig. **99:**1525–1533.
17. **Kitchen, S. G., Y. D. Korin, M. D. Roth, A. Landay, and J. A. Zack.** 1998. Costimulation of naive $CD8^+$ lymphocytes induces CD4 expression and allows human immunodeficiency virus type 1 infection. J. Virol. **72:**9054–9060.
18. **Kitchen, S. G., S. LaForge, V. P. Patel, C. M. Kitchen, M. C. Miceli, and J. A. Zack.** 2002. Activation of CD8 T cells induces expression of CD4, which functions as a chemotactic receptor. Blood **99:**207–212.
19. **Klein, M. R., C. A. van Baalen, A. M. Holwerda, S. R. Kerkhof Garde, R. J. Bende, I. P. M. Keet, J. K. M. Eeftinck-Schattenkerk, A. D. M. E. Osterhaus, H. Schuitemaker, and F. Miedema.** 1995. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. J. Exp. Med. **181:**1365–1372.
20. **Livingstone, W. J., M. Moore, D. Innes, J. E. Bell, P. Simmonds, J. Whitelaw,**

R. Wyld, J. R. Robertson, and R. P. Brettle. 1996. Frequent infection of peripheral blood CD8-positive T-lymphocytes with HIV-1. Lancet **348:**649–654.

21. **McBreen, S., S. Imlach, G. R. Scott, C. Leen, J. E. Bell, and P. Simmonds.** 2001. Preferential infection of the CD45RA$^+$ (naive) subset of CD8$^+$ lymphocytes by human immunodeficiency virus type 1 in vivo. J. Virol. **75:** 4091–4102.
22. **McMichael, A. J., and S. L. Rowland-Jones.** 2001. Cellular immune responses to HIV. Nature **410:**980–987.
23. **Ortolani, C., E. Forti, E. Radin, R. Cibin, and A. Cossarizza.** 1993. Cytofluorimetric identification of two populations of double positive (CD4$^+$,CD8$^+$) T lymphocytes in human peripheral blood. Biochem. Biophys. Res. Commun. **191:**601–609.
24. **Pantaleo, G., A. De Maria, S. Koenig, L. Butini, B. Moss, M. Baseler, H. C. Lane, and A. S. Fauci.** 1990. CD8$^+$ T lymphocytes of patients with AIDS maintain normal broad cytolytic function despite the loss of human immunodeficiency virus-specific cytotoxicity. Proc. Natl. Acad. Sci. USA **87:**4818–4822.
25. **Potter, S. J., D. E. Dwyer, and N. K. Saksena.** 2003. Differential cellular distribution of HIV-1 drug resistance in vivo: evidence for infection of CD8$^+$ T cells during HAART. Virology **305:**339–352.
26. **Robbins, G. K., M. M. Addo, H. Troung, A. Rathod, K. Habeeb, B. Davis, H. Heller, N. Basgoz, B. D. Walker, and E. S. Rosenberg.** 2003. Augmentation of HIV-1-specific T helper cell responses in chronic HIV-1 infection by therapeutic immunization. AIDS **17:**1121–1126.
27. **Rodrigo, A. G., P. C. Goracke, K. Rowhanian, and J. I. Mullins.** 1997. Quantitation of target molecules from polymerase chain reaction-based limiting dilution assays. AIDS Res. Hum. Retrovir. **13:**737–742.
28. **Rosenberg, E. S., M. Altfeld, S. H. Poon, M. N. Phillips, B. M. Wilkes, R. L. Eldridge, G. K. Robbins, R. T. D'Aquila, P. J. Goulder, and B. D. Walker.** 2000. Immune control of HIV-1 after early treatment of acute infection. Nature **407:**523–526.
29. **Semenzato, G., C. Agostini, L. Chieco-Bianchi, and A. De Rossi.** 1998. HIV load in highly purified CD8$^+$ T cells retrieved from pulmonary and blood compartments. J. Leukoc. Biol. **64:**298–301.
30. **Semenzato, G., C. Agostini, L. Ometto, R. Zambello, L. Trentin, L. Chieco-Bianchi, and A. De Rossi.** 1995. CD8$^+$ T lymphocytes in the lung of acquired immunodeficiency syndrome patients harbor human immunodeficiency virus type 1. Blood **85:**2308–2314.
31. **Sullivan, Y. B., A. L. Landay, J. A. Zack, S. G. Kitchen, and L. Al Harthi.** 2001. Upregulation of CD4 on CD8$^+$ T cells: CD4^{dim} CD8^{bright} T cells constitute an activated phenotype of CD8$^+$ T cells. Immunology **103:**270–280.
32. **van Baarle, D., S. Kostense, E. Hovenkamp, O. Ogg, N. Nanlohy, M. Callan, N. Dukers, A. McMichael, M. van Oers, and F. Miedema.** 2002. Lack of Epstein-Barr virus (EBV)- and HIV-specific CD27$^-$ CD8$^+$ T cells is associated with progression to viral disease in HIV infection. AIDS **16:**2001–2011.
33. **Yang, L. P., J. L. Riley, R. G. Carroll, C. H. June, J. Hoxie, B. K. Patterson, Y. Ohshima, R. J. Hodes, and G. Delespesse.** 1998. Productive infection of neonatal CD8$^+$ T lymphocytes by HIV-1. J. Exp Med. **187:**1139–1144.
34. **Zerhouni, B., J. A. Nelson, and K. Saha.** 2004. Isolation of CD4-independent primary human immunodeficiency virus type 1 isolates that are syncytium inducing and acutely cytopathic for CD8$^+$ lymphocytes. J. Virol. **78:**1243–1255.
35. **Zhang, J., A. Gupta, R. Dave, M. Yimen, B. Zerhouni, and K. Saha.** 2001. Isolation of primary HIV-1 that target CD8$^+$ T lymphocytes using CD8 as a receptor. Nat. Med. **7:**65–72.
36. **Zloza, A., Y. B. Sullivan, E. Connick, A. L. Landay, and L. Al Harthi.** 2003. CD8$^+$ T cells that express CD4 on their surface (CD4^{dim} CD8^{bright} T cells) recognize an antigen-specific target, are detected in vivo, and can be productively infected by T-tropic HIV. Blood **102:**2156–2164.

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First evidence of HIV infection of CD8 lymphocytes expressing CD4 during primary HIV-1 infection

Activated CD8 lymphocytes express CD4 generating a $CD8^{bright}CD4^{dim}$ phenotype that is susceptible to HIV infection both *in vitro* [1–3] and *in vivo* [4]. Here we demonstrate for the first time that CD8 lymphocytes in a patient with primary HIV infection express CD4 and are infected with HIV. This finding has implications for the immune control of HIV during primary infection, and for the generation of a long-lived viral reservoir.

The patient presented with a typical HIV seroconversion illness, consisting of fever, rash and lymphadenopathy approximately 4 weeks after HIV exposure through unprotected heterosexual intercourse. Primary HIV infection was confirmed by two weakly reactive HIV antibody tests with a negative Western blot (fourth generation HIV combo assay, Abbott Diagnostics, Abbott Park, Illinois, USA; fourth generation Vidas Duo assay, BioMerieux, Boulogne, France; and New LAV Blot 1, BioRad, Hercules, California, USA). HIV p24 antigen was positive (Vidas p24 antigen assay, BioMerieux) and could be neutralized with specific antiserum. The initial HIV viral load was 3.5×10^6 RNA copies/ml serum (Roche Cobas Monitor HIV-RNA assay, Roche Diagnostics, Mannheim, Germany) rising to 6.3×10^6 a week later, then dropping to 2×10^4 after one month.

Blood was drawn for this study during the phase of rising viraemia, 3 weeks after the onset of symptoms. The prevalence of $CD8^{bright}CD4^{dim}$ lymphocytes circulating in peripheral blood was determined on a FACSVantage flow cytometer (Becton Dickenson, Franklin Lake, New Jersey, USA) using fluorescent conjugated monoclonal antibodies: CD4-FITC (Molecular Probes, Paisley, UK), CD8beta-PE (Coulter Immunotech, Beckman Coulter, Inc. Miami, Florida) and CD3-cychrome (BD Biosciences). Of the total T lymphocytes (defined as CD3 expressing cells with forward and side scatter properties typical of lymphocytes) 26% were CD4 lymphocytes, 67% were CD8 lymphocytes, and 0.4% were $CD8^{bright}CD4^{dim}$ lymphocytes (Fig. 1a). This frequency of $CD8^{bright}CD4^{dim}$ lymphocytes was similar to levels found previously both in the general population [5] and in individuals with chronic HIV infection [6]. This single subject thus demonstrated CD4 cell expression on CD8 lymphocytes, but does not suggest that primary HIV infection leads to the exceptionally high levels of expression described for primary Epstein–Barr virus and cytomegalovirus infection [7].

The level of HIV infection of $CD8^{bright}CD4^{dim}$ and $CD8^{+}CD4^{-}$ negative lymphocytes was determined using a method previously validated in chronic HIV infection [4]. Briefly, highly purified populations of CD4 lymphocytes, CD8 lymphocytes expressing CD4 ($CD8\beta^{bright}CD4^{dim}$) and CD8 lymphocytes not expressing CD4 ($CD8\beta^{bright}CD4^{-}$) were isolated. The HIV-DNA copies per million cells in each of the isolated populations were determined by the extraction of DNA followed by quantitative polymerase chain reaction for HIV long terminal repeat (LTR) repeat DNA.

The HIV DNA load in CD4 lymphocytes was 697 (SE 225) HIV DNA copies per million CD4 lymphocytes, a frequency in keeping with results from a previous series of 16 subjects with primary HIV infection [8]. Interestingly, the level of infection of $CD8^{bright}CD4^{dim}$ lymphocytes was similar to that of CD4 lymphocytes at 523 (SE 160) HIV DNA copies per million $CD8^{bright}CD4^{dim}$ lymphocytes, with only two HIV DNA copies per million cells being attributable to CD4 lymphocyte contamination. No HIV DNA was found in the CD8 lymphocytes not expressing CD4 (Fig. 1b) [9].

The frequency of infection of CD4 lymphocytes circulating in the blood during primary HIV infection substantially underrepresents the level of HIV infection occurring in lymph nodes and mucosal lymphoid tissue [10]. The same is likely to be true of CD8 lymphocytes, particularly as the susceptible ($CD8^{bright}CD4^{dim}$) phenotype is generated on antigen-specific activation in lymph tissue. Therefore, the proportion of circulating CD8 lymphocytes infected with HIV observed in this study may reflect much greater frequencies of infection in lymph tissue.

Events during primary HIV infection have a major influence on the course of disease, with poor viraemic control being prognostic of rapid progression [11]. CD8 lymphocytes are vital in the control of initial viraemia [12,13], and thus any compromise of CD8 lymphocyte function at this stage could have a profound effect on prognosis. HIV infection of $CD8^{bright}CD4^{dim}$ lymphocytes leads to the downregulation of CD4 expression, the deregulation of IFN-γ and FAS ligand expression, and decreased effector function [14]. The infection of $CD8^{bright}CD4^{dim}$ lymphocytes demonstrated here could thus significantly compromise the primary anti-HIV CD8 lymphocyte response.

In addition, the infection of CD8 lymphocytes has implications for the generation of a long-lived HIV reservoir. Unlike CD4 lymphocytes where activation is

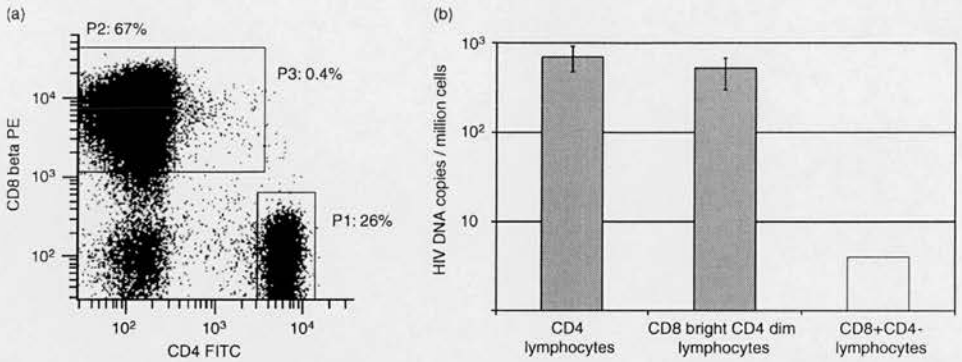

Fig. 1. The extent of CD4 expression on CD8 lymphocytes and the level of HIV infection of these cells are shown for the study subject with primary HIV infection. (a) Expression of CD4 and CD8β on T lymphocytes (gated on light scatter characteristics and CD3 expression from peripheral blood mononuclear cells). The proportions of CD4 lymphocytes (P1), CD8 lympocytes not expressing CD4 (P2) and $CD8^{bright}CD4^{dim}$ lymphocytes (P3) are shown. (b) HIV DNA load attributable to CD4 lymphocytes, $CD8^{bright}CD4^{dim}$ lymphocytes and $CD8^{+}CD4^{-}$ lymphocytes. Filled bars represent detected attributable HIV DNA loads, the unfilled bar represents undetectable viral DNA, and half the lower limit of detection is given. Error bars show standard errors estimated using the program 'Quality' [9].

rapidly followed by apoptosis, a significant proportion of activated CD8 lymphocytes differentiate into memory cells [15]. While resting, these memory cells are invisible to immune surveillance, can form a long-lived refuge for HIV [16], and therefore could contribute to the failure of antiretroviral therapy to achieve complete HIV clearance, even when treatment is started soon after seroconversion [17].

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References

1. Sullivan YB, Landay AL, Zack JA, Kitchen SG, Al Harthi L. **Upregulation of CD4 on CD8+ T cells: $CD4^{dim}CD8^{bright}$ T cells constitute an activated phenotype of CD8+ T cells.** *Immunology* 2001; **103**:270–280.
2. Flamand L, Crowley RW, Lusso P, Colombini Hatch S, Margolis DM, Gallo RC. **Activation of CD8(+) T lymphocytes through the T cell receptor turns on CD4 gene expression: implications for HIV pathogenesis.** *Proc Natl Acad Sci U S A* 1998; **95**:3111–3116.
3. Kitchen SG, Korin YD, Roth MD, Landay A, Zack JA. **Costimulation of naive CD8(+) lymphocytes induces CD4 expression and allows human immunodeficiency virus type 1 infection.** *J Virol* 1998; **72**:9054–9060.
4. Cochrane A, Imlach S, Leen C, Scott G, Kennedy D, Simmonds P. **High levels of human immunodeficiency virus infection of CD8 lymphocytes expressing CD4 *in vivo*.** *J Virol* 2004; **78**:9862–9871.
5. Blue ML, Daley JF, Levine H, Schlossman SF. **Coexpression of T4 and T8 on peripheral blood T cells demonstrated by two-color fluorescence flow cytometry.** *J Immunol* 1985; **134**: 2281–2286.
6. Zloza A, Sullivan YB, Connick E, Landay AL, Al Harthi L. **CD8+ T cells that express CD4 on their surface ($CD4^{dim}CD8^{bright}$ T cells) recognize an antigen-specific target, are detected in vivo, and can be productively infected by T-tropic HIV.** *Blood* 2003; **102**:2156–2164.
7. Ortolani C, Forti E, Radin E, Cibin R, Cossarizza A. **Cytofluorimetric identification of two populations of double positive (CD4+, CD8+) T lymphocytes in human peripheral blood.** *Biochem Biophys Res Commun* 1993; **191**:601–609.
8. Karlsson AC, Birk M, Lindback S, Gaines H, Mittler JE, Sonnerborg A. **Initiation of therapy during primary HIV type 1 infection results in a continuous decay of proviral DNA and a highly restricted viral evolution.** *AIDS Res Hum Retroviruses* 2001; **17**:409–416.
9. Rodrigo AG, Goracke PC, Rowhanian K, Mullins JI. **Quantitation of target molecules from polymerase chain reaction-based limiting dilution assays.** *AIDS Res Hum Retroviruses* 1997; **13**:737–742.
10. Douek DC, Picker LJ, Koup RA. **T cell dynamics in HIV-1 infection.** *Annu Rev Immunol* 2003; **21**:265–304.
11. Lyles RH, Munoz A, Yamashita TE, Bazmi H, Detels R, Rinaldo CR, *et al.* **Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS Cohort Study.** *J Infect Dis* 2000; **181**:872–880.
12. Koup RA, Safrit JT, Cao YZ, Andrews CA, Mcleod G, Borkowsky W, *et al.* **Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.** *J Virol* 1994; **68**:4650–4655.
13. Musey L, Hughes J, Schacker T, Shea T, Corey L, McElrath MJ. **Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection.** *N Engl J Med* 1997; **337**:1267–1274.

14. Kitchen SG, Jones NR, LaForge S, Whitmire JK, Vu BA, Galic Z, *et al.* **CD4 on CD8(+) T cells directly enhances effector function and is a target for HIV infection.** *Proc Natl Acad Sci U S A* 2004; **101**:8727–8732.
15. Homann D, Teyton L, Oldstone MB. **Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory.** *Nat Med* 2001; **7**:913–919.
16. Potter SJ, Dwyer DE, Saksena NK. **Differential cellular distribution of HIV-1 drug resistance *in vivo*: evidence for infection of CD8+ T cells during HAART.** *Virology* 2003; **305**:339–352.
17. Rosenberg ES, Altfeld M, Poon SH, Phillips MN, Wilkes BM, Eldridge RL, *et al.* **Immune control of HIV-1 after early treatment of acute infection.** *Nature* 2000; **407**:523–526.

Human herpes virus 8 in HIV and non-HIV infected patients with pulmonary arterial hypertension in France

Kaposi's sarcoma-associated herpesvirus or human herpesvirus 8 (HHV8) is a vasculotrope virus associated with Kaposi's sarcoma, HIV-associated Castleman's disease and primary effusion lymphoma. Other associations, in particularly multiple myeloma, have been clearly disproved by additional analysis after initial positive reports [1]. Cool *et al.* [2] have recently demonstrated the presence of HHV8 in the lungs of patients with pulmonary arterial hypertension (PAH) ascertained by immunochemistry with antibody directed against latency-associated nuclear antigen 1 (LANA-1) and polymerase chain reaction assay to detect the viral cycline gene of HHV8. In that study, evidence of HHV8 was found in 10 out of 16 patients with idiopathic PAH, in one out of three patients with PAH related to HIV infection, and in none of 12 patients with PAH related to other conditions. The authors suggested a role for HHV8 in the development of PAH [2]. Later, the presence of HHV8 antibodies, against a structural HHV8 protein called K8.1 and against LANA-1, was tested in plasma samples from non-HIV-infected German patients with PAH, but no difference was found between patients and controls [3]. In addition, HHV8-DNA sequences were not detected by polymerase chain reaction in nine PAH lungs studied in Japan, indicating that other factors than HHV8 are more likely to be the cause of PAH in that population [4]. Moreover, whereas the geographical distribution of Kaposi's sarcoma mirrors HHV8 prevalence, it seems unlikely for PAH in which no ethnic predisposition has been observed to date. As Mediterranean countries have higher prevalence rates of HHV8 infection, we studied a cohort of French patients with PAH in order to test whether there were possible links between HHV8 and PAH in this country. The aim of the study was to evaluate the prevalence of HHV8 antibodies in plasma samples from HIV and non-HIV-infected patients with PAH.

Ninety-three patients with PAH were enrolled in the study: 47 with idiopathic or familial PAH, 34 with PAH related to HIV infection, and 12 with PAH associated with other conditions. Antibodies directed against LANA-1 were detected using an immunofluorescence assay on the primary effusion lymphoma BC-3 cell line, and the positive samples were confirmed using an immunofluorescence assay that detects antibodies to HHV8 lytic antigens (KSHV IgG IFA; Biotrin, Dublin, Ireland).

HHV8 antibodies were detected in one out of 47 [2.1%; confidence interval (CI) 0–6.3%] patients with idiopathic or familial PAH and in two out of 12 (16.7%; CI 0–38.7%) patients with PAH related to other conditions. In the 34 patients with PAH related to HIV infection, eight had HHV8 antibodies (23.5%; CI 9.3–37.8%). These results indicate that the prevalence of antibodies for HHV8 in HIV-infected and non-HIV-infected patients with PAH is similar to that previously reported for corresponding individuals without PAH. The seroprevalence of HHV8 in healthy individuals in France has been estimated at 2%, and between 22 and 35% among HIV-infected individuals from the United States and northern Europe using LANA tests [5,6]. This suggests that there is no trend in favour of an increased serological evidence of HHV8 infection in PAH in HIV-infected patients or in non-HIV-infected patients. In addition, a reduced immunological response to viruses has never been reported in PAH, and there is no scientific basis supporting the hypothesis of a defective humoral response to HHV8, which could explain the low prevalence of HHV8-specific antibodies in plasma samples of PAH patients. Our present results are based on serological evidence of HHV8 infection, and one may hypothesize that HHV8 infection could be restricted to patients' lungs. However, such compartmentalized HHV8 infection without serological evidence has not been reported to date. We conclude that there is no evidence in favour of an association between PAH and HHV8 in a French cohort of HIV-seropositive and seronegative patients.

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